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(54) Title: PEPTIDES AND ANTI-SENSE PEPTIDES WITH BROAD NEOPLASTIC SPECIFICITY

(57) Abstract

An analogue peptide comprises the variable regions of the light or heavy chains of an antibody of a first species selectively binding to a tumor antigen, such as a carcinoma antigen, has 1 to 46 amino acids of the framework regions per chain substituted with amino acids such as those present in equivalent positions in antibodies of a species other than the first species, or fragments thereof comprising 1 to 3 variable region CDRs per chain and optionally flanking regions thereof of 1 to 10 or more amino acids, alone or with an N-terminal fragment of 1 to 10 or more amino acids, combinations or mixtures thereof. The polypeptide may also comprise an effector agent and/or be glycosylated, and is presented as a composition with a carrier. The analogue peptides are used in diagnostic kits for neoplasms such as carcinomas and methods for in vivo imaging and treating a primary or metastasized neoplasm such as a carcinoma, and in vitro diagnosing a neoplasm, ex vivo purging neoplastic cells from a biological fluid. RNAs and DNAs encode the analogue peptide, and a hybrid vector carrying the nucleotides and transfected cells express the peptides and a method produces the analogue peptide. An anti-idiotype polypeptide comprises polyclonal antibodies raised against an antineoplastic antibody or the analogue peptide of this invention, monoclonal antibodies thereof, Fab, Fab', (Fab')$_2$, CDR, variable region, or analogues or fragments thereof, combinations thereof with an oligopeptide comprising a TRP trimer, tandem repeats thereof, or combination or mixtures thereof. An anti-idiotype hybrid polypeptide with an effector agent and the anti-idiotype polypeptide, an anti-neoplastic vaccine, an anti-neoplastic vaccination kit, a method of vaccinating against neoplasms such as carcinomas and a method of lowering the serum concentration of a circulating antibody or polypeptide are provided.

PEPTIDES AND ANTI-SENSE PEPTIDES WITH BROAD NEOPLASTIC SPECIFICITY

BACKGROUND OF THE INVENTION

Field of the invention

This invention relates to the in vitro and in vivo diagnosis, immunization, and therapy of neoplastic tumors, particularly carcinomas, by

5   means of specifically targeted analogue peptides comprising amino acid sequences encompassing the complementarity determining regions (CDRs) of a different species, and analogues of the variable (F_v) region of anti-carcinoma antibodies, among others. The carcinoma specific peptides for use in one species, e.g., humans, are provided as a single amino acid chain having the

10   specificity of F_v regions obtained in different species, e.g., murine antibody F_v regions of the light or heavy chains, or as paired chains. These peptides are provided either by themselves or bound to other molecules such as synthetic polymers or oligopeptides resulting in sequences of mixed species, and more particularly analogues of human/non-human chimeric antibodies or other

15   polymeric constructs. The analogue peptides comprise sequences derived from the variable regions of heterologous antibodies specific for, e.g., human carcinoma antigens that elicit a lesser immunological response in humans than the whole heterologous antibodies. The anti-idiotype polypeptides and their analogues are suitable for immunizing humans or other animals against

20   carcinoma. Polynucleotide segments encoding the analogue peptide and anti-idiotype polypeptides, and hybrid vectors and transfected host cells carrying the segments are useful for preparing the peptides disclosed herein.

Description of the Background

Carcinomas result from the carcinogenic transformation of cells of

25   different epithelia. Two of the most damaging characteristics of carcinomas are their uncontrolled growth and their ability to create metastases in distant sites of the host, particularly a human host. It is usually these distant metastases that cause serious consequences to the host, since frequently the primary carcinoma may be, in most cases, removed by surgery. The treatment of metastatic

30   carcinomas, that are seldom removable, depends on irradiation therapy and systemic therapies of different natures. The systemic therapies currently include, but not fully comprise, chemotherapy, radiation, hormone therapy, different immunity-boosting medicines and procedures, hyperthermia and systemic monoclonal antibody treatment. The latter can be labeled with

35   radioactive elements, immunotoxins and chemotherapeutic drugs.

Radioactively labeled monoclonal antibodies were initially used with success in lymphomas and leukemia, and recently in some carcinomas. The concept underlying the use of labeled antibodies is that the labeled antibody will specifically seek and bind to the carcinoma and, the radioactive element, through

its decay, will irradiate the tumor in situ. Since radioactive rays travel some distance in tumors it is not necessary that every carcinoma cell bind the labeled antibody. The specificity of the monoclonal antibodies will permit a selective treatment of the tumor while avoiding the irradiation of innocent by-stander

5   normal tissues, that could be dose limiting. Chemotherapy produces serious toxic effects on normal tissues, making the chemotherapy of carcinomas less than desirable, and the use of radiolabeled monoclonal antibodies a valid alternative.

    Non-human antibodies raised against human epitopes have been used for

10 the diagnosis and therapy of carcinomas as is known in the art. Also known are the methods for preparing both polyclonal and monoclonal antibodies. Examples of the latter are BrE-2, BrE-3 and KC-4 (e.g., US patent Nos. 5,077,220; 5,075,219 and 4,708,930..

    The KC-4 murine monoclonal antibody is specific to a unique antigenic

15 determinant, the "antigen", and selectively binds strongly to neoplastic carcinoma cells and not to normal human tissue (U.S. Patent No. 4,708,930 to Coulter). The antigen appears in two forms in carcinoma cells, only the smaller of these forms being expressed in the cell membrane. The larger form appears only in the cytoplasm and has an approximate 490 Kdalton molecular weight

20 (range of 480,000-510,000). The second form occurs at a higher density of expression, is found both in the cytoplasm and the membrane of carcinoma cells and has an approximate 438 Kdalton molecular weight (range of 390,000-450,000) as determined by gel electrophoresis with marker proteins of known molecular weights. Labeled KC-4 was applied to the diagnosis and medical

25 treatment of various carcinomas, particularly adenocarcinoma and squamous cell carcinoma regardless of the human organ site of origin.

    The BrE-3 antibody (Peterson et al., Hybridoma 9:221 (1990); US patent No. 5,075,219) was shown to bind to the tandem repeat of the polypeptide core of human breast epithelial mucin. When the mucin is deglycosylated, the

30 presence of more tandem repeat epitopes is exposed and the binding of the antibody increases. Thus, antibodies such as BrE-3 bind preferentially to neoplastic carcinoma tumors because these express an unglycosylated form of the breast epithelial mucin that is not expressed in normal epithelial tissue. This preferential binding combined with an observed low concentration of epitope for

35 these antibodies in the circulation of carcinoma patients, such as breast cancer patients, makes antibodies having specificity for a mucin epitope highly effective for carcinoma radioimmunotherapy. A ^{80}Y-BrE-3 radioimmunoconjugate proved highly effective against human breast carcinomas transplanted into nude mice. Human clinical studies showed the ^{80}Y-BrE-3 radioimmunoconjugate to

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considerably reduce the size of breast tumor metastases without any immediate toxic side effects. Moreover, an ^{111}In-BrE-3 radioimmunoconjugate was successfully used for imaging 15 breast cancer patients, providing excellent tumor targeting in 13 out of 15 of the patients. Out of all the breast tumor
5 metastases occurring in another study, 86% were detected by ^{111}In-BrE-3. Unfortunately, 2 to 3 weeks after treatment, the patients developed a strong human anti-murine antibody (HAMA) response that prevented further administration of the radioimmunoconjugate. The HAMA response, which is observed for numerous murine monoclonal antibodies, precludes any long-term
10 administration of murine antibodies to human patients. Similarly, other heterologous antibodies, when administered to humans, elicited similar antibody responses. The anti-heterologous human response is, thus, a substantial limiting factor hindering the successful use of heterologous monoclonal antibodies as therapeutic agents, which could, otherwise, specifically annihilate breast
15 carcinomas, causing little or no damage to normal tissue and having no other toxic effects.

Chimeric antibodies are direct fusions between variable domains of one species and constant domains of another. Murine/human chimeric antibodies prepared from other types of B cells binding to other types of antigenic
20 determinants have been shown to be less immunogenic in humans than whole murine antibodies. These proved to be less immunogenic but still in some cases an immune response is mounted to the rodent variable region framework region (FR). A further reduction of the "foreign" nature of the chimeric antibodies was achieved by grafting only the CDRs from a rodent monoclonal into a human
25 supporting framework prior to its subsequent fusion with an appropriate constant domain, (European Patent Application, Publication No. 239,400 to Winter; Riechmann, et al., Nature 332:323-327 (1988)). However, the procedures employed to accomplish CDR-grafting often result in imperfectly "humanized" antibodies. That is to say, the resultant antibody loses avidity (usually 2-3 fold,
30 at best).

The ligand binding characteristics of an antibody combining site are determined primarily by the structure and relative disposition of the CDRs, although some neighboring residues also have been found to be involved in antigen binding (Davies, et al., Ann. Rev. Biochem. 59:439-473 (1990)).
35 The technologies of molecular biology have further expanded the utility of many antibodies by allowing for the creation of class switched molecules whose functionality has been improved by the acquisition or loss of complement fixation. The size of the bioactive molecule may also be reduced so as to increase the tissue target availability of the antibody by either changing the class

from an IgM to an IgG, or by removing most of the heavy and light chain constant regions to form an F$_v$ antibody. Common to all of these potentially therapeutic forms of antibody are the required complementary determining regions (CDRs), which guide the molecule to its ligand, and the framework

5   residues (FRs) which support the CDRs and dictate their disposition relative to one another. The crystallographic analysis of numerous antibody structures revealed that the antigen combining site is composed almost entirely of the CDR residues arranged in a limited number of loop motifs. The necessity of the CDRs to form these structures, combined with the appreciated hypervariability of their

10   primary sequence, leads to a great diversity in the antigen combining site, but one which has a finite number of possibilities. Thus, its hypermutability and the limited primary sequence repertoire for each CDR would suggest that the CDRs derived for a given antigen from one species of animal would be the same derived from another species. Hence, they should be poorly immunogenic, if at

15   all, when presented to a recipient organism.

Accordingly, there is still need for a product of high affinity and/or specificity for carcinoma antigens suitable for the detection and therapy of carcinomas which elicits a lesser antibody response than whole non-human antibodies or chimeric antibodies containing, for instance the entire non-human

20   variable region.

SUMMARY OF THE INVENTION

This invention relates to an analogue peptide and its glycosylated derivative which specifically and selectively bind to the human mammary fat globule (HMFG) antigen and to an antigen found on the surface or in the

25   cytoplasm of tumor cells such as carcinoma cells or that is released by the cells, the analogue peptide consisting essentially of at least one variable region of the light or heavy chains of an antibody of a first species having affinity and specificity for an antigen found on the surface or the cytoplasm of a carcinoma cell or released by the cell, wherein preferably about 1 to at least 46 amino acids

30   in the FR are substituted per chain with amino acids selected from the group consisting of amino acids present in equivalent positions in antibodies of a species other than the first species, or fragments thereof comprising 1 to 3 CDRs per chain or 1 to 3 CDRs plus flanking regions thereof, each of about 1 to at least 10 amino acids, alone or with an N-terminal fragment of about 1 to at least

35   10 amino acids, combinations thereof or combinations thereof with other variable regions or analogues thereof, wherein each analogue peptide is operatively linked to at least one other peptide or analogue thereof, or mixtures thereof.

Also provided herein are a fusion protein and a hybrid polymer comprising the analogue of the invention, the corresponding DNAs encoding them, hybrid vector thereof, transfected host thereof, and RNA.

This invention also encompasses a method of producing an analogue
5   peptide or hybrid analogue peptide by recombinant technology, in vitro methods of diagnosing and immunohistochemistry of tissue slices, an ex vivo method of purging neoplastic cells, and in vivo methods for imaging and therapy of neoplasias such as carcinomas.

Also disclosed herein are anti-idiotype polypeptides comprising polyclonal
10 antibodies raised against the analogue peptide of this invention, analogues thereof, monoclonal antibodies thereof, fragments thereof selected from the group consisting of Fab, Fab', (Fab')$_2$, CDRs, variable regions and analogues thereof as described above, combinations thereof operatively linked to one another, an anti-carcinoma vaccine, a vaccination kit, a method of vaccinating
15 against neoplasias including carcinomas, and a method of lowering the serum concentration of circulating anti-tumor antibody with the anti-idiotype polypeptide of this invention.

Other objects, advantages and features of the present invention will become apparent to those skilled in the art from the following discussion.

20   DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention arose from a desire by the inventors to improve on antibody technology suitable for use in diagnostic, prognostic, vaccine and therapeutic applications in the field on neoplasmas and cancer. The present invention will be described for application to humans. However, it is also
25 suitable for use in other species. The monoclonal antibodies obtained up to the present time have been prepared by fusing immortalized cell lines with B-cells of non-human origin such as murine, rat, rabbit, goat, and the like. Many of these hybridomas can produce large quantities of monoclonal antibodies that have desirable binding properties such as high affinity and/or specificity and selectivity
30 for carcinoma antigens of a species, e.g., for human carcinoma antigens. However, in general, antibodies from other species, e.g., non-human antibodies, may only be administered once to a subject of a predetermined species, e.g., humans, due to the detrimental effects they produce. This is true for most heterologous antibodies being administered to mammalian animals. For example,
35 the repeated administration of murine antibodies to a human subject elicits a strong human anti-murine antibody (HAMA) response, which precludes their further utilization as therapeutic agents in humans. These non-human antibodies initiate an immediate adverse reaction in many human patients and are, thus,

rendered ineffective for further administration as therapeutic agents. In some cases, non-human-human chimeric antibodies and non-human CDR "grafted"-human antibodies may have lower affinity and/or specificity for their antigens than the corresponding non-human antibody. On the other hand, human

5   monoclonal hybridoma cell lines have not been very stable and have, therefore, not been suitable for the large scale, repeated production of monoclonal antibodies.

The present invention is applicable to the manufacture of analogue peptides having a high number of amino acids in the respective positions found

10 in the endogenous antibodies, and where at least the CDRs are of another xenogeneic species.

The present inventors, thus, have undertaken the preparation of antitumor non-human CDRs and non-human variable regions of antibodies, having affinity and specificity for an antigen found on the surface or the

15 cytoplasm of a human neoplastic cell such as a carcinoma cell or that is released by the cell, wherein about 1 to 46 amino acids in the framework region (FR) are substituted per chain with amino acids present in equivalent positions in other human antibodies, or fragments thereof comprising 1 to 3 CDRs per chain or 1 to 3 CDRs per chain plus flanking regions thereof, each of about 1 to 10 or more

20 amino acids, alone or plus an N-terminal fragment of about 1 to 10 or more amino acids, to lower or even circumvent the anti-xenogeneic response. To preserve substantial binding specificity in the molecules intended for use in humans, the present invention utilizes CDRs and/or analogues of varying lengths of the variable regions of light and/or heavy chains of antibodies of other species

25 such as murine, rat, rabbit, goat, equine, primate, bovine, and guinea pig antibodies, among others. Heterologous human CDRs and/or analogues of their variable regions may be utilized are intended for use in other species.

The present inventors have found, surprisingly, that these analogue antibody fragments substantially preserve the binding, specificity and selectively

30 characteristics of the whole non-human antibody while eliciting a lesser detrimental immunological response. However, the simple preservation of the binding region of an antibody does not by itself ensure that the binding characteristics of the antibody will be maintained. Antibodies are glycopolypeptides that are folded into specific conformations. When the

35 glycoside portion of the molecule or portions of the amino acid sequence are perturbed or excised, the folding pattern of the molecule may be perturbed. Thus, any deletion or modification of the sequence of an antibody must be made taking into consideration that its folding-dependent properties may be diminished or even obliterated if the folding is substantially affected, even though the amino

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acid sequences involved in the binding of the antigen are preserved.

The present inventors selected the following strategy for the preparation and manufacture of the analogue and hybrid peptides of this invention. The cDNAs that encode the variable chains of an antibody may be obtained by

5   isolation of mRNA from a hybridoma cell and reverse transcription of the mRNA, amplification of the cDNA by polymerase chain reaction (PCR) and insertion of the DNA into a vector for optional sequencing, and for restriction enzyme cutting. The cDNAs encoding the CDRs or variable chain (F_v) region fragments of the light (V_L) and heavy (V_H) chains of an antibody having affinity and

10   specificity for a carcinoma cell antigen may be reverse transcribed from isolated mRNA. The cDNAs encoding the CDRs may then be ligated to other segments encoding neighboring sequences, and the variable region cDNAs may then be modified with predesigned primers used to PCR amplify them or synthesized de novo, cloned into a vector optionally carrying DNA sequences encoding, e.g.,

15   a constant region(s), optionally sequenced, and then transfected into a host cell for expression of the analogue gene product. The binding specificity characteristics of the analogue peptides may then be determined and compared to those of the whole antibodies.

X-ray crystallographic studies demonstrate that the framework

20   structures of the F_v of different antibodies assume a canonical structure regardless of the species of origin, amino acid sequence, or ligand specificity. This is generally taken as evidence that the ligand-binding characteristics of an antibody combining site are determined primarily by the structure and relative disposition of the CDRs, although some neighboring framework residues may

25   also be involved in antigen-binding. Thus, if the fine specificity of an antibody is to be preserved, its CDR structures, and probably some of the neighboring residues, their interaction with each other and with the rest of the variable domains, must also be maintained. These crystallographic studies point to the possible need for retaining most, if not all, of the many interior and inter-domain

30   contact residues since the structural effects of replacing only a few of them cannot be predicted.

While at first the necessity of keeping these amino acids might seem to defeat the goal of decreasing immunogenicity by "humanization", the actual number of amino acids that must be retained is usually small because of the

35   striking similarity between human and murine variable regions. Moreover, many, if not most, of the retained amino acids possess side chains that are not exposed on the surface of the molecule and, therefore, may not contribute to its antigenicity. Clearly, it is most of the exposed amino acids that are good candidates for substitution since it is these amino acids that are exposed to the

immunological environment of a mammal and may form epitopes of increased immunogenicity.

The challenge in humanizing the variable regions of a non-human antibody, e.g., a murine antibody, thus begins with the identification of the

5   "important" xenogeneic amino acids. "Important" amino acids are those, for example, that are involved in antigen binding, contact the CDRs and the opposite chains, and have buried side-chains. Ideally, these residues might be identified from a well characterized three-dimensional structure. However, when direct structural data are not available, it is, fortunately, still possible to predict the

10   location of these important amino acids by analyzing other related antibody structures, especially those whose variable light and heavy regions belong to the same class. The classes of variable regions can be determined from their amino acid sequence.

A method by which these important amino acids are identified has been

15   described for the case of the amino acids with buried side chains by Padlan, E.A. (Padlan, E.A., "A Possible Procedure for Reducing the Immunogenicity of Antibody Variable Domains While Preserving Their Ligand-Binding Properties", Molecular Immunology, 28:489-494 (1991)). In the present case, various antibody variable region structures were compared using a computer program

20   that determines the solvent accessibility of the framework residues as well as their contacts with the opposite domain as described by Padlan, E.A. (1991), supra. Surprisingly, a close examination of the fractional solvent accessibility reveals a very close similarity in the exposure patterns of the V_H and the V_L domains. Put in simple terms, regardless of the particular antibody in question,

25   and of its amino acid sequence, the buried residues occupy similar relative positions in most antibodies.

A similar analysis can be done by computer modeling, to determine which amino acids contact the CDRs and which contact the opposite domain. At this point, the Fab structures that are currently in the Protein Data bank

30   (Bernstein,F.C., et al., J. Mol. Biol. 112:535-542 (1977)) may be examined to determine which FRs are probably important in maintaining the structure of the combining site. Thus, after a close inspection of many high resolution three-dimensional structures of variable regions, the positions of all important framework amino acids, that is, those that contact the CDRs, the opposite

35   domain, and those whose side chains are inwardly pointed, may be tabulated. Keeping these amino acids, as well as those from the CDRs, and finally those FR amino acids that may be involved in ligand binding, should insure to a great extent the preservation of affinity. The precise identification of FR amino acids that are involved in ligand-binding cannot be generalized since it varies for

differe... antibodies. Nevertheless, conservative dec...ns can be made to preserve the amino acids located in FR that have a high probability of contacting the antigen. These regions are located immediately adjacent to the CDRs and at the N-terminus of both chains, because the surfaces of these regions are

5 contiguous with the CDR surfaces.

Surprisingly, it is possible to keep all of these important amino acids in a heterologous humanized antibody and still increase dramatically the similarity with a human consensus sequence. That is, the final number of amino acids with murine identities differing from human identities that are kept is typically small.

10 This is usually possible because human frameworks that are similar to the murine frameworks, especially at the positions of the important amino acids, can be found. This is because many of the important amino acids have the same identities in both murine and human antibodies.

All the amino acids that are determined to be not important by the

15 method described above may be completely replaced by their corresponding human counterparts. The surface of the finally humanized antibody should look very much like that of a human antibody except for the antigen binding surfaces. The original shape of those binding surfaces, however, is maintained by leaving the internal composition of the antibody intact, preserving inter-domain contacts

20 and by keeping very few key amino acids that contact the CDRs.

a)   Choosing the Best Human Framework to
       Use in the "Humanization" of an Antibody
       When Its Structure Is Known
       At the present time, there are 11 Fab structures for which the atomic

25 coordinates are known and have been placed in the Protein Data Bank as shown in Table 1 below, 2 from human and 9 from murine antibodies.

Table 1:   Fab Structures for Which Coordinates
            are in the Protein Data Bank

	ANTIBODY	RESOLUTION (A)	R-VALUE	PDB CODE
HUMAN:	NEWM	2.0	0.46	3FAB
	KOL	1.9	0.189	2FB4
MURINE:	McPC603	2.7	0.225	1MCP
	J539	1.95	0.194	2FBJ
	HyHEL-5	2.54	0.245	2HFL
	HyHEL-10	3.0	0.24	3HFM
	R19.9	2.8	0.30	1F19
	4-4-20	2.7	0.215	4FAB
	36-71	1.85	0.248	6FAB
	B1312	2.8	0.197	1IGF
	D1.3	2.5	0.184	1FDL

The contacts between side chains in the variable domains of the 11 Fabs have been collected and are presented in Tables 2 to 4 below. The framework (FR) amino acids (aa's) in the V_L domains that contact CDRs are listed in Table 2 below.

5 Table 2: V_L Framework Residues That Contact CDR Residues
in Fabs of Known Three-Dimensional Structure

POSITION	JS39	McPC603	HyHEL-10	HyHEL-5	R19.9	ANTIBODY 4-4-20	36-71	8I3I2	D1.3	NEWM	KOL
1	GLU(2)	ASP(5)	ASP(10)	ASP(3)		ASP(8)	ASP(4)		ASP(11)		
2	ILE(11)	ILE(15)	ILE(17)	ILE(13)	ILE(5)	VAL(9)	ILE(20)	VAL(9)	ILE(10)	SER(3)	
3		VAL(3)	VAL(2)	VAL(3)	GLN(2)	VAL(2)	GLN(2)	LEU(5)			VAL(2)
4	LEU(7)	MET(6)	LEU(6)	LEU(10)	MET(9)	MET(13)	MET(7)	MET(6)	MET(7)	LEU(4)	LEU(6)
5		THR(1)			THR(1)	THR(2)	. .			THR(1)	
7								THR(4)			
22								SER(6)			CYS(1)
23	CYS(1)	CYS(1)	CYS(2)	CYS(2)	CYS(1)	CYS(1)	CYS(1)				TRP(2)
35	TRP(3)	TRP(2)	TRP(4)		TRP(2)		TRP(6)	TRP(4)	TRP(4)	TRP(1)	
36	TYR(12)	TYR(16)	TYR(8)	TYR(10)	TYR(22)	TYR(13)	TYR(15)	TYR(8)	TYR(14)	TYR(13)	TYR(11)
45					LYS(12)	LYS(5)					
46	PRO(3)	LEU(6)	LEU(4)	ARG(15)	LEU(5)	VAL(14)	LEO(5)	LEU(10)	LEU(6)	LEU(2)	LEU(6)
48	ILE(1)	ILE(1)	ILE(1)				ILE(3)	ILE(2)	VAL(1)		ILE(1)
49	TYR(28)	TYR(29)	LYS(13)	TYR(12)	TYR(40)	TYR(22)	TYP(22)	TYR(16)	TYR(25)		TYR(25)
58	VAL(3)	VAL(3)	ILE(1)	VAL(6)	VAL(6)	VAL(5)	VAL(4)	VAL(5)	VAL(1)		VAL(6)
60		ASP(1)				ASP(2)		ASP(4)			ASP(2)
62			PHE(1)			PHE(1)	PHE(1)			LYS(2)	LYS(11)
66	.								SER(1)		
67		SER(3)							THR(1)	SER(1)	
69		THR(3)	THR(3)			THR(5)	THR(1)	THR(4)		SER(2)	
70		ASP(2)			ASP(1)		ASP(6)				ALA(4)
71	TYR(14)	PHE(23)	PHE(17)	TYR(17)	TYR(24)	PHE(17)	TYR(17)	PHE(19)	TYR(16)	ALA(3)	CYS(1)
88	CYS(1)		CYS(2)		CYS(1)	CYS(1)	CYS(1)	CYS(1)	CYS(2)		PHE(7)
98	PHE(8)	PHE(8)	PHE(10)	PHE(5)	PHE(8)	PHE(4)	PHE(8)	PHE(14)	PHE(14)	PHE(3)	

Those FR in the V_H domains that contact CDRs are listed in Table 3
10 below.

Table 3: V_H Framework Residues That Contact CDR Residues
in Fabs of Known Three-Dimensional Structure

POSITION	JS39	McPC603	HyHEL-10	HyHEL-5	R19.9	ANTIBODY 4-4-20	36-71	8I3I2	D1.3	NEWM	KOL
1							GLU(3)				
2	VAL(11)	VAL(3)	VAL(6)		VAL(1)		VAL(7)	VAL(3)	VAL(12)		VAL(9)
4	LEU(2)	LEU(3)	LEU(5)		LEU(1)	LEU(1)	LEU(1)	LEO(1)	LEU(1)		LEU(1)
24		THR(2)	VAL(6)			ALA(1)					
27	PHE(3)	PHE(2)		TYR(4)	TYR(1)	PHE(26)	TYR(4)	PHE(4)	PHE(4)	TYR(1)	PHE(3)
28	ASP(9)	THR(5)		TYR(3)	THR(6)	THR(4)	THR(3)			SER(1)	ILE(2)
29	PHE(4)	PHE(4)		PHE(10)	PHE(7)	PHE(13)	PHE(6)	LEU(1)			PHE(4)
30			THR(2)			THR(6)	SER(7)			ASP(6)	
34							TRP(2)	.	VAL(1)	VAL(2)	VAL(1)
36					VAL(1)						ARG(3)
37		VAL(1)									
38	ARG(1)	ARG(2)	ARG(4)	LYS(2)	LYS(1)	ARG(4)	LYS(2)	ARG(1)	VAL(1)	VAL(2)	VAL(1)
40				ARG(1)							ARG(3)
44	GLU(3)	GLO(4)	GLU(1)	GLU(2)	GLU(3)	GLU(4)	GLU(9)		GLU(1)		GLU(1)
47	TRP(21)	TRP(29)	TYR(20)	TRP(21)	TRP(13)	TRP(10)	TRP(21)	TRP(23)	TRP(13)	TRP(22)	TRP(15)
48	ILE(1)	ILE(1)	MET(6)	ILE(12)	ILE(13)	VAL(1)	ILE(9)	VAL(3)	LEU(1)	ILE(1)	VAL(1)
49		ALA(1)				ALA(2)		ALA(2)			ALA(2)
67			ARG(1)	ALA(1)		AAG(3)		AAG(2)		ARG(2)	ARG(1)
66	PHE(4)	PHE(10)	ILE(9)	ALA(1)		PHE(11)	THR(5)	PHE(12)	LEO(6)	VAL(2)	PHE(10)
68		ILE(1)			THR(1)	THR(1)					THR(2)
69	ILE(6)	VAL(6)	ILE(6)	PHE(12)	LEU(5)	ILE(20)	LEU(6)	ILE(11)	ILE(8)	MET(4)	ILE(9)
71	ARG(7)	ARG(16)	ARG(21)	ALA(1)	VAL(4)	AAG(6)	VAL(6)	ASG(3)	LYS(4)		ARG(9)
72	ASN(1)	THR(3)				ASP(3)					
73	LEU(4)	LEU(7)	TYR(9)	ALA(1)	ALA(1)	VAL(2)	ALA(1)	LEO(4)	VAL(4)	PHE(5)	LEU(9)
80						LEU(1)					
82		LEU(2)							MET(1)	LEU(1)	
86						ASP(2)					
92				CYS(1)			CYS(1)		CYS(1)	ALA(1)	ALA(2)
93	ALA(4)	ALA(5)		LEU(1)		THR(3)	ALA(1)	TES(5)	ALA(4)	ALA(1)	ALA(2)
94	ARG(28)	ARG(24)	ASN(11)	HIS(2)	ARG(30)		ARG(23)	ARG(14)	ARG(30)	ARG(22)	ARG(27)
101	TRP(5)	TRP(9)			TRP(2)	TRP(2)	TRP(5)			TRP(4)	TRP(4)

10

.he FR amino acids, that contact the oppo e domain and which presumably are the ones mainly responsible for the quaternary structure of the F_v domains are listed in Table 4 below.

Table 4:　　Framework Residues That Contact Framework Residues in the Opposite Domain in Fabs of Known Three-Dimensional Structure

IN V_L:

POSITION	J539	McPC603	HyHEL-10	HyHEL-5	ANTIBODY R19.9	4-4-20	36-71	8132	D1.3	NEWM	KOL
36	TYR(3)	TYR(4)	TYR(3)	TYR(5)		TYR(11)	TYR(7)	TYR(1)	TYR(7)		TYR(5)
38	GLN(10)	GLN(4)	GLN(9)	GLN(5)	GLN(5)	GLN(3)	GLN(6)	GLN(12)	GLN(6)	GLN(7)	GLN(8)
43	SER(7)	PRO(1)	SER(8)	SER(5)	THR(3)			SER(3)	SER(2)	ALA(5)	ALA(1)
44	PRO(10)	PRO(14)	PRO(8)	PRO(11)		PRO(7)	ILE(20)	PRO(16)	PRO(16)	PRO(7)	PRO(13)
46	PRO(3)										
85		MET(2)						VAL (1)		ASP(12)	
87	TYR(6)	TYR(4)	PHE(6)	TYR(2)			PHE(5)	TYR(10)	TYR(8)	TYR(6)	TYR(6)
98	PHE(11)	PHE(8)	PHE(7)	PHE(12)	PHE(12)	PHE(12)	PHE(8)	PHE(13)	PHE(12)	PHE(10)	PHE(13)
100		ALA(2)									

IN V_H:

POSITION	J539	McPC603	HyHEL-10	HyHEL-5	ANTIBODY R19.9	4-4-20	36-71	8132	D1.3	NEWM	KOL
37	VAL(4)		ILE(2)	VAL(1)	VAL(4)	VAL(2)	VAL(1)	VAL(2)	VAL(4)	VAL(1)	VAL(4)
39	GLN(10)	GLN(4)	LYS(8)	GLN(3)	GLN(5)	GLN(3)	GLN(6)	GLN(10)	GLN(6)	GLN(4)	GLN(7)
43			ASN(4)		GLN(7)			LYS(6)		ARG(19)	
44		ARG(2)									
45	LEU(13)	LEU(12)	LEU(8)	LEU(14)		LEU(8)	LEU(11)	LEU(13)	LEU(14)	LEU(11)	LEU(16)
47	TRP(1)		TYR(2)		TRP(2)				TRP(3)	TRP(2)	
91	TYR(6)	TYR(4)	TYR(3)	TYR(8)	PHE(3)	TYR(2)	PHE(4)	TYR(3)	TYR(5)	TYR(3)	
103	TRP(11)	TRP(13)	TRP(16)	TRP(11)	TRP(4)	TRP(18)	TRP(24)	TRP(22)	TRP(19)	TRP(8)	TRP(15)
105	GLN(5)										

The buried, inward-pointing FR amino acids in the V_L domains, i.e., those which are located in the domain interior, are listed in Table 5 below.

Table 5:　　Inward-Pointing, Buried Framework Residues in the V_L of Fabs of Known Three-Dimensional Structure

POSITION	J539	McPC603	HyHEL-10	HyHEL-5	ANTIBODY R19.9	4-4-20	36-71	8132	D1.3	NEWM	KOL
2	ILE	ILE	ILE	ILE	VAL	VAL	VAL	ILE		ILE
4	LEU	MET	LEU	LEU	MET	MET	MET	MET	MET	LEU	LEU
6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
11	THR	LEU	LEU	MET	LEU	LEU	LEU	LEU	LEU	VAL	ALA
13	ALA	VAL	VAL	ALA	VAL	ALA	ALA	ALA	VAL	VAL	VAL
19	VAL	VAL	VAL	VAL	VAL	ALA	VAL	ALA	VAL	ILE	ILE
21	ILE	MET	LEU	MET	ILE	ILE	ILE	ILE	ILE	CYS	CYS
23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP
37	GLN	GLN	GLN	GLN	GLN	LEU	GLN	LEU	GLN	GLN	GLN
43	TRP	LEU	LEU	TRP	LEU	LEU	LEU	LEU	LEU	LEU	LEU
48	ILE	ILE	ILE	ILE	VAL	ILE	VAL	ILE		ILE
49									PHE	
58	VAL	VAL	ILE	VAL	VAL	VAL	VAL	VAL	VAL	VAL
61	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG
62	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	TYR	ALA	ALA
71	TYR	PHE	PHE	TYR	TYR	PHE	TYR	TYR	TYR	LEU	LEU
73	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	ILE	LEU
75	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
78	MET	VAL	VAL	MET	LEU	VAL	LEU	VAL	LEU	LEU	LEU
82	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
83									PHE	
84	ALA	ALA		ALA	ALA		ALA		ALA	TYR	THR
86	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
88	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
102	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	VAL
104	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	VAL
106	LEU	ILE	ILE	ILE					ILE	VAL	VAL

Those in the V_H domain are listed in Table 6 below.

Table .   Inward-Pointing, Buried Framework Re ues in
the V$_H$ of Fabs of Known Three-Dimensional Structure

POSITION	J539	McPC603	HyHEL-10	HyHEL-5	R19.9	4-4-20	36-71	8I3I2	0I.3	NEWM	KOL
2	VAL	VAL	VAL				VAL	VAL	VAL	LEU	VAL.
4	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU.
6	GLU	GLU	GLU	GLN	GLU	GLU	GLN	GLU	GLU	GLN	GLN
9		PRO									
11	VAL	VAL	VAL	MET	VAL	VAL	VAL	VAL	VAL	VAL	VAL
18	LEU	LEU	LEU	VAL	VAL	MET	VAL	LEU	LEU	LEU	LEU
20	LEU	LEU	LEU	ILE	MET	LEU	MET	LEU	ILE	LEU	LEU
22	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
24	ALA	THR	VAL	ALA	ALA	ALA	ALA	ALA	VAL	VAL	SER
27	PHE	PHE	ASP	TYR	TYR	PHE	TYR	PHE	PHE	THR	PHE
29	PHE	PHE	ILE	PHE	PHE	PHE	PHE	PHE	LEU	PHE	PHE
36	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP
38	ARG	ARG	ARG	LYS	LYS	ASG	LYS	ARG	ARG	ARG	ARG
40						SER					
46		GLU	GLU	GLU		VAL	ILE	VAL	LEU	ILE	VAL
48	ILE	ILE	MET	ILE		ILE	VAL	ALA	ALA		ALA
49		ALA				ALA		ARG			ARG
66	LYS	ASG	ARG	LYS				ARG	ASG	ARG	PHE
67	PHE	PHE	ILE	ILA	TYR	PHE	THR	PHE	LEU	VAL	ILE
69	ILE	VAL	ILE	PHE	LEU	ILE	LEU	ILE	ILE	MET	ARG
71	ARG	ARG	ARG	ALA	VAL	ARG	VAL	ARG	LYS	VAL	
76					SER						LEU
78	LEU	LEU	TYR	ALA	ALA	VAL	ALA	LEU	VAL	PHE	LEU
80	LEU	LEU	LEU	MET	MET	LEU	MET	LEU	LEU	LEU	MET
82	MET	MET	LEU	LEU	LEU	MET	LEU	LEU	MET	VAL	LEU
82C	VAL	LEU	VAL	LEU	LEU	LEU	LEU	ASP	ASP	ASP	ASP
86	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ALA	ALA	ALA	
88	ALA	ALA	ALA	ALA	ALA			TYR	TYR	TYR	TYR
90	TYR	TYR	TYR	TYR	TYR	TYR	TYR	CYS	CYS	CYS	CYS.
92	CYS	CYS	CYS	CYS	CYS	CYS		ARG	ARG	ARG	ASG
94	ARG	ARG	ASN	HIS	ARG		THR	THR	THR	SER	THR
107	THR	THR		THR	THR	THR	LEU	LEU	LEU	VAL	VAL
109	VAL	VAL	VAL	LEU	LEU	VAL	LEU	LEU	LEU	VAL	VAL
111	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL

From the above, it may be seen that

(1) There are many FR amino acids that either contact the CDRs or the opposite domain, or are found in the domain interior.

(2) These FR amino acids, which could influence the structure of the combining site, and thus the antigen-binding characteristics of an antibody, are different from antibody to antibody.

It is obvious from these results that no one structure can serve as the perfect and sole basis of all "animalization", or in the present example "humanization", protocols. In fact, to "humanize" the 9 murine antibodies shown in Table 1 above by CDR-grafting with a view to preserving their ligand-binding properties, the FR amino acids listed in Table 2 to 6 above would have to be retained.

A search through the tables of immunoglobulin sequences (Kabat et al., "Sequences of Proteins of Immunological Interest", 5th Ed. US Dept. of Health and Human Service, NIH Publication No.91-3242 (1991)), shows that human variable domain sequences are known that already have most of the FR amino acids that need to be preserved as shown in Table 7 below.

Table 7: Human Antibodies that are Most Similar in
Sequence to Murine Antibodies of Known
Three-Dimensional Structure

ANTIBODY DOMAIN		MOST SIMILAR HUMAN SEQUENCE
HyHEL-10	VH	S8P2'CL (77/112)
	VH FRAMEWORK	1SP1'CL, HL1'CL (62/87)
	VH IMPT	S8P2'CL, Ab26'CL, C6B2'CL (28/38)
	VL	IARC/BL41'CL (73/107)
	VL FRAMEWORK	IARC/BL41'CL (59/80)
	VL IMPT	IARC/BL41'CL (30/37)
HyHEL-5	VH	ND'CL (74/116)
	VH FRAMEWORK	783c'CL, X17115'CL (63/87)
	VH IMPT	21/28'CL, S1P1'CL, 783c'CL, 8E10'CL, AND, KAS, NEI'CL, X17115'CL (25/37)
	VL	HF2-1/17'CL, KAS (65/105)
	VL FRAMEWORK	HF2-1/17'CL (57/80)
	VL IMPT	BI, DEN, HF2-1/17'CL, KUE, REI, WALKER'CL, WIL(-) (27/36)
R19.9	VH	21/28'CL (73/119)
	VH FRAMEWORK	21/28'CL, S1P1'CL, AND, LS2'CL, NEI'CL (60/87)
	VH IMPT	21/28'CL, 8E10'CL, LS2'CL (28/38)
	VL	WALKER'CL (78/107)
	VL FRAMEWORK	RZ (62/80)
	VL IMPT	REI, WALKER'CL (33/36)
4-4-20	VH	30P1'CL (77/116)
	VH FRAMEWORK	2P1'CL, 3D6'CL (65/87)
	VH IMPT	4B4'CL, H26'CL (36/41)
	VL	RPM1-6410'CL (91/112)
	VL FRAMEWORK	GM-607-'CL (68/80)
	VL IMPT	CUM, FR, NIH (33/36)
J539	VH	30P1'CL, Vh38C1.10'CL (81/118)
	VH FRAMEWORK	18/2'CL, 30P1'CL, M43 (71/87)
	VH IMPT	38P1'CL, S6P1'CL, M72, M74 (36/40)
	VL	PA (62/105)
	VL FRAMEWORK	LEN, WEA (53/80)
	VL IMPT	BI, DEN, KUE, REI, WALKER'CL, WIL(-) (26/35)
McPC603	VH	H72 (81/120)
	VH FRAMEWORK	4G12'CL, Ab18'CL, M72 (70/87)
	VH IMPT	56P1'CL, M72, M74, RF-SJ2'CL (36/42)
	VL	FK-001'CL, LEN (91/113)
	VL FRAMEWORK	LEN (70/80)
	VL IMPT	LEN (38/42)
36-71	VH	21/28'CL (74/119)
	VH FRAMEWORK	21/28'CL, S1P1'CL, 783c'CL, AND'CL, NEI'CL, X17115'CL, (61/87)
	VH IMPT	21/28'CL, 8E10'CL (28/38)
	VL	AG (76/105)
	VL FRAMEWORK	RZ (63/80)
	VL IMPT	REI, RZ, WALKER'CL (34/37)

Table   Human Antibodies that are Most Simil
Sequence to Murine Antibodies of Known
Three-Dimensional Structure (Cont'd)

B13I2	VH	S6Pi'CL (83/119)
	VH FRAMEWORK	4B4!CL, 4G12'CL, H26'CL, H72, RF-SJ2'CL, Vh38C1.10'CL
		(68/87)
	VH IMPT	S6P1'CL, H72, H74, RF-SJ2'CL (37/39)
	VL	RPH1-6410'CL (86/112)
	VL FRAMEWORK	GM-607-'CL (69/80)
	VL IMPT	CUM, NIM (36/39)
D1.3	VH	C6B2'CL (72/116)
	VH FRAMEWORK	C6B2'CL (62/87)
	VH IMPT	H60'CL (32/37)
	VL	BR (75/107)
	VL FRAMEWORK	KF2-1/17'CL (64/80)
	VL IMPT	J06'CL, BI, DEN, EU, KUE, PA, REI, WALKER'CL, WIL(-) (J:

5   These human sequences are not necessarily those which are most similar
to the murine antibodies, overall or in the framework regions only, but rather,
those that possess the largest number of important amino acids in common, the
latter sequences being included in Table 7 above.

The number of murine amino acids that still need to be retained in order
10   to have all the important FR amino acids in the "humanized" or analogue versions
of the murine antibodies, as shown in Table 7 above, ranges from 21 (for
HyHEL-5:12 in V_H and 9 in V_L) to 5 (for B1312:2 in V_H and 3 in V_L). These are
not very many amino acids, considering that the resulting "humanized" or
analogue molecules will probably retain most or all their ligand-binding
15   characteristics. It is possible that there exist other human sequences that are
even more similar to these murine domains that are not included in the
compilation of Kabat, et al. (1991), supra. When more sequences become
available these may also be incorporated to improve the pool of basic data
available.

14

b)   Choosing the best human framework to
      use in the "humanization" of an antibody
      when its structure is not known
      In the absence of a three-dimensional structure, the identification of the

5   FR amino acids that are crucial to maintain the combining site structure is not

easily done. Nevertheless, some proposals may be made from the data shown

in Tables 2 to 6 above that have been collected in Tables 8 and 9 below for the

V_L and V_H domains.

Table 8:   Framework Residues in V_L That Probably
10   Need to Be Preserved in Order to Reproduce the
             Ligand Properties of the Original Antibody

```
                          CDR1
J539     EI.L.Q....T.A.....V.I.C  aase---------avaalb HYQQ....SP.PWIY
k:c?C603 GIVHTQ....L.V.....V.H.C  kbaqallnagnqknfle HYQQ....PP.LLIY
HyHEL-10 OIVL.Q....L.V.....V.L.C  caaq--------aignalh HYQQ....SP.LLIK
HyHEL-5  OIVL.Q....H.A.....V.H.C  aaae--------avnymy HYQQ....SP.AWIY
R19.9    .IQHTQ....L.A.....V.I.C  caaq------disaylh HYLQ.....PKVLIY
4-4-20   DVVHTQ....L.V.....A.I.C  caaq-alvbaqgntylr HYQQ......I.LLIY
36-71    OIQH.Q....L.A.....V.I.C  caaq-------dinnfln HYQQ.....I.LLIY
81312    .VLH.QF...L.V.....A.ISC  caag-tilladgdtyla WYLQ....SP.LLIY
D1.3     DI.H.Q....L.A.....V.I.C  caaq--------nihnyla HYQQ....SP.LLVY

         CDR2                                    CDR3
J539     eisklas .V..RF.........Y.L.I.H...D.A.YYC qqvtyplit F...T.L.L.
k:c?C603 gastras .V.DRF....S.TOF.L.I..V...D.A.XYC qndhayplt F.A.T.L.I.
HyHEL-10 yasqsis .I..RF......T.F.L.I..V...D..HYFC qqanavpyt f...T.L.I.
HyHEL-5  dtaklas .V..RF.......Y.L.I..H...D.A.YYC qqvgr-npt F...T.L.I.
R19.9    ytarlha .V..RF......DY.L.I..L...D.ATI:C qqgattprt F...7:L...
4-4-20   kvsnrfs .V.DRF......T.F.L.I..V...D.A.YFC qqgnalprt F...T.L.I.
36-71    ttsrsqs .V..RF......TOY.L.I..L...D.A.YFC fqgahwppt R--.T.C.I.
81312    kvsnrfs .V.DRF......T.F.L.I..V...D..VYYC qbfwstprt F...7.L...
D1.3     ytttlad .V..RF....S.T.Y.L.I..L...DF..YYC qbfwstprt F...7.L...
```

Table 9:   Framework Residues in V_H That Probably
            Need to Be Preserved in Order to Reproduce the
            Ligand Properties of the Original Antibody

15   From Tables 8 and 9 above, it may be seen that many of the important

FR amino acids flank the CDRs. Among these flanking positions are most of the

FR amino acids that are involved in the contact with the opposite domain as shown in Table 4 above, and many of those which are in contact with the CDRs as shown in Tables 2 and 3 above. Moreover, almost all of the FR amino acids that have been observed to participate in the binding to antigen (Amit, A.G., et

5　al., Science 233:747-753 (1986); Sheriff, et al., P.N.A.S. (USA) 82:1104-1107 (1987); Padlan, E.A., et al. ,P.N.A.S. (USA) 86:5938-5942 (1989); Tulip, et al., Cold Spring Harbor Symp. Quant. Biol. 54:257-263 (1989); Bentley, et al., Nature (London) 348: 254-257 (1990)), are in these flanking regions. Thus, during "animalization" or "humanization" or formation of the analogue peptides,

10　not just the CDRs are retained, but also some of the residues immediately adjacent to the CDRs. This provides a better chance of retaining more of the ligand-binding properties of the original antibody. The likelihood of retaining the antigen binding properties of the original antibody is even greater if the first few amino acids in the NH_2-termini of both chains are also retained, since some of

15　them are found to be in contact with CDRs as shown in Tables 2 and 3 above. Further, Tables 8 and 9 above also show many other framework positions that are deemed structurally important in all the cases examined here. The xenogeneic residues at those positions should probably be retained as well.

　　　　　Alternatively, it may possible to reduce immunogenicity, while preserving

20　antigen-binding properties, by simply replacing those exposed residues in the framework regions which differ from those usually found in human antibodies (Padlan, E.A. (1991), supra). This would "humanize" the surface of the xenogeneic antibody while retaining the interior and contacting residues which influence its antigen-binding characteristics. The judicious replacement of

25　exterior residues should have little, or no, effect on the interior of the domains, or on the interdomain contacts. For example, the solvent accessibility patterns of the F_vs of J539, a murine IgA (κ) and of KOL, a human IgG1 (λ) have been found to be very similar (Padlan, E.A. (1991), supra).

　　　　　At present, more than 35 different Fab structures have been elucidated

30　by X-ray diffraction analysis, although atomic coordinates for only 11 are currently in the Protein Data Bank as shown in Table 1 above. Most of the available structures have been analyzed to only medium resolution, some having been refined to only a limited extent. Eventually, atomic coordinates for more and better-refined structures will become available, so that the "important" FRs

35　will be more easily assessed. This will improve the theoretical predictive record of the present method for determining the best mode for the analogue peptides.

　　　　　As already indicated above, the specificity of an antibody depends on the CDR structures and sometimes, on some neighboring residues as well. These structures, in turn, depend on contacts with framework amino acids and on the

interaction of the V_L and V_H domains. Thus, to ensure the retention of binding affinity, not only the CDR residues must be preserved, but also those FRs that contact either the CDR's or the pposite domain, as well as all buried residues, which give shape to the variable domains.

5   This design of the humanized versions of murine antibodies is reached in stages as follows.

      1- Choice of a xenogeneic model of known structure.
      2- Choice of the target species FR.
      3- Identification of xenogeneic/target species differences.
10   4- Identification of important xenogeneic amino acids.

(1) Choice of a xenogeneic model of known structure

      The V_H and V_L domains of an antibody of desired specificity are classified according to Kabat et al.(1991), supra. Then, an antibody of the same species may be chosen, whose structure has been determined, and whose

15 variable regions belong to the same classes and subclasses. Modeling the xenogeneic antibody in question to such structure ensures maximal chance for success. This, however, is not absolutely necessary since the relative positions of the important amino acids do not vary considerably even in variable regions of different classes. Thus, with less than a perfect match this method may still

20 be applied to design the analogues of this invention. Once the xenogeneic model is chosen, it may be applied to identify the locations of important residues in the xenogeneic antibody to be animalized (humanized). Tables 2, 3, 4, 5, 6, 8 and 9 indicate the positions of the important amino acids in several antibodies whose structures have been determined to a high resolution level.

25 **(2) Choice of the target species FR**

      The target species framework should, ideally, be a consensus framework. That is, one that has a maximum number of amino acids in common with all human frameworks of the same class. This is important, because, the goal of humanization is to avoid an immunological response against the engineered

30 analogue peptide.

      The target species framework that is chosen is that which shares the greatest number of important amino acids with the original xenogeneic antibody. Thus, in choosing the target species (human) FR, the similarity between the important amino acids is more important that the overall similarity.

35   In practice, the sequences of the xenogeneic variable chains are aligned with the consensus sequences from all variable region classes of the target species and the number of differences in the amino acids that must be retained from the xenogeneic species are scored. The human consensus sequence(s) that

score(s) the lowest number of differences is (are) then chosen. These are the best analogue peptide candidates. Others with low numbers that are higher than the above may also be suitable, and are placed in a reserve pool, and so forth.

If there are too many differences in the chosen framework (e.g., more than 16),

5 then the same alignment procedure using all tabulated human sequences may be repeated in order to find a specific human framework whose similarity with the xenogeneic sequence is maximized at the positions of the important amino acids. Thus, most preferably, the target species FR should be a consensus sequence. Next preferable would be a framework of a common target species (human)

10 antibody, and finally, the framework of any target species (human) antibody.

(3) Identification of xenogeneic target species differences

The xenogeneic sequences are then aligned with the target species sequences and the positions of all amino acids that differ in the murine and in the human frameworks are tabulated. Such a table contains the maximum

15 number of amino acids that can be changed toward the full "animalization" ("humanization") of the xenogeneic antibody (see, Tables 31 and 32 below). If all those changes were to be made, a so-called CDR-grafted antibody would be obtained. That is, only the original CDRs would be retained from the murine antibody. In some cases, possibly, such CDR-grafted antibody may maintain the

20 original binding affinity. In most instances, however, the affinity of a CDR-grafted antibody would be considerably less than that of the original xenogeneic antibody. In order to maximize the chances for conserving the original affinity, the identities of all important amino acids must be preserved.

(4) Identification of important xenogeneic amino acids

25 If the outlined approach to animalization (humanizing) an antibody is followed strictly, the amino acids that are correspondingly important in the model xenogeneic antibody chosen in step 1 are retained. In a more preferred approach, however, the amino acids that have been shown to occupy important positions in other antibodies of the same species or of the target species may also be

30 retained and are therefore taken out from the group of candidates to be mutated. This preferred approach may be particularly appropriate when there is a chance that the amino acids in question could make contacts with the CDRs or with the opposite chains. Once the important xenogeneic amino acids are identified, the DNA sequence may be mutagenized to change all other amino acids, which for

35 the most part occupy exposed positions.

The present method is exemplified for a murine antibody humanized with the intent of diminishing or avoiding a HAMA response upon its administration

to humans. Murine and human antibodies, whose three-dimensional structures have been deduced to a high degree of resolution, were utilized as guidance in the choice of the amino acids to be substituted in order to humanize the particular murine antibody utilized. The method, however, may be applied more

5　generally to transform antibodies from one species into a less immunogenic form to be administered to a second species, provided that adequate three-dimensional models are available for antibodies from those species. Information on other murine antibodies from a Data Bank was used in the exemplary disclosure provided below to modify the BrE-3 and anti-KC-4 murine-human

10　chimeric antibodies with human amino acids. Similarly, antibodies of other species besides murine may also be utilized, their CDRs and other amino acids preserved and those amino acids not considered "important" replaced with human amino acids. Similarly, the above approach may be applied to the preparation of "animalized" antibodies for any animal species. This may be

15　attained by substituting amino acids of the antibody target species into an antibody of another species in accordance with this invention.

　　　　Various peptide structures, such as CDRs, and analogue antibodies, Fab, Fab', (Fab')$_2$, and variable fragments having a desired specificity, may be constructed and optionally bridged via a linker. In addition, one or more of the

20　peptides may be attached to one or more effector agent(s) or bridged via a linker. Multiple antibody, variable regions, Fab, Fab', (Fab')$_2$, CDRs and the like, and combinations thereof, may also be constructed and bridged via linkers or attached to one or more effector agents such as are described below.

　　　　The cDNAs encoding the analogue variable regions of an antibody of a

25　desired specificity may be cloned into a vector, optionally containing sequences encoding constant regions or fragments thereof, enzymes, neuropeptides, other peptide transmitters, toxins, hormones, operative conjugation regions, cytokines, lymphokines and the like, optionally under the same promoter. Although this is the cloning strategy utilized in the exemplary disclosure of this invention, other

30　methods known in the art may also be utilized such as co-expression and the like. In the exemplary disclosure provided herein, the anti-BrE-3 and anti-KC-4 murine-human chimeric antibodies specifically binding to human mammary mucin and carcinoma cells was constructed by joining the DNAs of the anti-BrE-3 or anti-KC-4 murine variable domain to a human constant domain (an effector

35　agent) cloned into a hybrid vector, and the product expressed by transfecting the vector into myeloma cells. The variable regions of the chimeric antibody were modified at the DNA level to obtain an analogue or "humanized" chimeric polypeptide. The modifications to the variable regions of the peptides may either be conducted by PCR amplification with primers that are custom tailored to

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Table 10: Alternative Amino Acids for V_L Chain (Cont.)

	BrE3	KC4	Most Preferred Analogue	Preferred Analogue	Not Preferred Analogue
FR2					
35	W	W			
36	F	F	YL(F)	IVHN	
37	L	L	Q	WLVKRTHDE	
38	Q	Q			IFMCATSDN
39	K	K	R		WIPMCA
40	S	P	P(S)	FLKARTGQ	
41G					IYFPMCT
42	Q	Q			WYVCD
43	S	S	P		WYFKMHDNEQ
44	P	P			WYKCRGHDQ
45	K	Q	EQR(K)		YPCGHD
46	L	L	RV		KCD
47	L	L	V	WILMTSN	
48	I	I		FPLVMTS	
49	Y	Y	S		PLVMA
FR3					
57	G	G		WVTSGDNEQ	
58	V	V		IYFLVMATQ	
59	P	P	S		
60	D	D	N		WFMCR
61	R	R	T		
62	F	F			
63	S	S	T	IYPLKARSG	
64	G	G	D		
65	S	S			
66	G	G			YCHDQ
67	S	S	A		VKMCRHN
68	E	G	GD(E)	VMCARSGQ	
69	T	T			WPMCE
70	D	D			WIFPMCR
71	F	F			WKMTEQ
72	T	T			WLMCGHNEQ
73	L	L			
74	K	K	NLRE		WLMCHD
75	I	I	L		
76	S	S	IT		LVMC
77	R	R	S		WYFLKCHQ
78	V	V	ALI		WYFKCRHNE
79	E	E	KGQ		WVKM
80	A	A	P		WLKM
81	E	E		ILVKMAGDNE	
82	D	D			
83	L	V	MV(L)		WCRH
84	G	G		LVARTSG	
85	V	I	IM(V)		WFKCQ
86	Y	Y			
87	F	Y	YL(F)	IMSHE	
88	C	C			

Table 11: Alternative Amino Acids for V$_H$ Chain (Cont'd.)

VL	BrE3	KC4	Most Preferred Analogue	Preferred Analogue	Not Preferred Analogue
FR2					
36	W	W			
37	V	V		WIFLVMATGQ	
38	R	R			YFS
39	Q	Q			FPVMCA
40	S	A	VA(S)		WY
41	P	P	TS	PLVARTSHNEQ	
42	E	G	G(E)		YPLKMCTHN
43	K	K			WIYLVCS
44	G	G	SR		WIFMCH
45	L	L			
46	E	E	Q		FPMCRTD
47	W	W			PMARNQ
48	V	V	STG		YPKCARTHNQ
49	A	A			WFPKCRHNQ
FR3					
66	R	R			WYLVMCATSDE
67	F	F			WYPKMHNEQ
68	T	T	IS		WYPGE
69	I	I			WYKCHNQ
70	S	S	L		WVMCHDEQ
71	R	R			WYFCHDQ
72	D	D	N	VKRTSGHDE	
73	D	N	N(D)		WYFLC
74	S	S	A	FPLVTGDN	
75	K	K	EN		NPCGD
76	S	N	NTRK(S)		WFPLMCE
77	R	T	TNIVSM(R)		WFC
78	V	L	LA(V)		WKCRNE
79	Y	Y	FH		WPG
80	L	L			
	WYLKCARGHEQ				
81	Q	Q	E		WYFPC
82	M	M			YPCTGHDEQ
82a	I	N	SDN(I)		WMQ
82b	S	S	IR		WYFPLMHQ
82c	L	L		PLVMAGE	
83	R	R	EKT		WILCH
84	A	A	SPVTI		WKCDEQ
85	E	E	D	WIYFPLMCRG	
86	D	D			
87	T	T	M		WLVMCNE
88	G	A	A(G)		
89	L	V	ITVM(L)		WYPKGEQ
90	Y	Y	HF		
91	Y	Y			WIPLVKMAGQ
92	C	C			
93	T	A	ASHAV(T)		WIFR
94	G	R	R(G)	TSDQLAW	
FR4					
103	W	W	A		
104	G	G	YH		
105	Q	Q	THR		IYFLVMCDQ
106	G	G			
107	T	T	AQ		
108	L	L	STGM		WYCHDE

suitable for imaging tumors in vivo. A CDR attached to an effector peptide may be synthesized chemically or recombinantly encoded in a DNA segment. Such small molecules have great tumor penetration and extremely rapid clearing properties when compared to larger antibody fragments. In some cases, it is

5   more convenient to produce these small molecules by chemical synthesis, as is known in the art, rather than by fermentation. In many cases, these small peptides are completely non-immunogenic and an immune response, such as the HAMA response, is altogether avoided. Also preferred are 2 and 3 CDR units per chain operatively linked to one another by 1 to 10 or more amino acids and up

10   to the entire inter-CDR segment length as positioned in the variable regions.

Heavy and light chain analogue variable regions may be obtained individually or in V_H/V_L pairs, or attached to an effector peptide such as a constant region(s) or portions thereof, a drug, an enzyme, a toxin, a whole antibody, or any other molecule or radioisotope. The fragments of the analogue

15   variable regions may be synthesized chemically as is known in the art or from the DNA segments encoding the non-human variable regions. This may be attained by PCR amplification of the DNA with primers synthesized to contain the desired mutation(s) as is known in the art. Similarly, the fragments encoding analogue variable regions may be synthesized chemically or obtained by established

20   cloning methods of restriction digestion, ligation, mutagenesis, and the like, as is known in the art.

There are advantages to using the different molecular variants of the analogue peptide depending on the specific applications for which they are intended, some of which are listed below.

25   a)   Smaller molecules penetrate target tissues more efficiently and are cleared from the body much more rapidly than larger molecules.
      b)   Single chain molecules can be manipulated and synthesized more efficiently that multiple chain molecules.
      c)   Many of these variants can be synthesized efficiently and

30   inexpensively in bacteria, including the non-glycosylated analogues.
      d)   Bi-functional or multifunctional molecules may carry polypeptide effectors, such as enzymes, toxins, radioisotopes, drugs, and other molecules, to a target tissue.

The following list encompasses exemplary analogue peptides of the

35   invention engineered with molecules derived from antibodies or antibody fragments. These analogue peptides, among others, are suitable for the practice of this invention. A more extensive list of polypeptide constructs may be found in O'Kennedy, R., and Roben, P. (O'Kennedy, R., and Roben, P., "Antibody Engineering: an Overview", Essays Biochem. (England) 26:59-75 (1991)).

40   The analogue peptides and hybrid peptides of this invention encompass CDRs and/or analogue variable regions, monoclonal antibodies, antibody

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fragments such as Fab, Fab', (Fab')$_2$, and fragments thereof, CDRs, constant regions, single or multiple-domain and catalytic fragments, bi-functional or multifunctional combinations thereof, enzymes, peptide hormones, molecules such as drugs and linkers, transmitters, and toxins, among others. These are
5   suitable for imaging, therapy, and diagnostics.

Single-Chain Antigen-Binding Polypeptides
     A method for constructing single chain antigen-binding polypeptides has been described by Bird et al. (Bird, R.E., et al., Science 242:243-246 (1988); Bird, R.E., et al., Science 244:409 (1989)). Single Chain F_v (scF$_v$ or sF$_v$) are
10   single chain analogue peptides containing both V_L and V_H with a linker such as a peptide connecting the two chains (V_L-linker-V_H). The engineering may be done at the DNA level, in which case knowledge of the sequence is required. These analogue peptides have the conformational stability, folding, and ligand-binding affinity of single-chain variable region immunoglobulin fragments and may be
15   expressed in E. coli. (Pantoliano, M.V., et al., Biochem. (US) 30:10117-25 (1991)). The peptide linker binding the two chains may be of variable length, for example, about 2 to 50 amino acid residues, and more preferably about 12 to 25 residues, and may be expressed in E. coli. (Pantoliano, M.V., et al. (1991), supra). An analogue peptide such as an scF$_v$ may be expressed and prepared
20   from E. coli and used for tumor targeting. The clearance profiles for scF$_v$ in some situations fragments are advantageous relative to those of normal antibodies, Fab, Fab' or (Fab')$_2$ fragments. (Colcher, D., et al., J. Natl. Cancer Inst. 82:1191-7 (1990)). Another type of analogue peptide comprises a V_H-linker-V_L and may have about 230 to 260 amino acids. A synthetic gene using E. coli
25   codons may be used for expression in E. coli. A leader peptide of about 20 amino acids, such that of Trp LE may be used to direct protein secretion into the periplasmic space or medium. If this leader peptide is not naturally cleaved, the sF$_v$ analogue peptide may be obtained by acid cleavage of the unique asp-pro peptide bond placed between the leader peptide and the sF$_v$-encoding region
30   (Houston, J.S., et al., "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain F$_v$ Analogue Produced in E. coli.", PNAS (USA) 85 (16):5879-83 (1988)). The construction, binding properties, metabolism and tumor targeting of the single-chain F$_v$ analogue peptides derived from monoclonal antibodies may be conducted as previously
35   described (Milenic, D.E., et al., Cancer Res. (US) 51 (23 pt1):6363-71 (1991); Yokota, et al., "Rapid Tumor Penetration of a single-chain F$_v$ and Comparison with Other Immunoglobulin Forms", Cancer Res. (US) 52(12):3402-8 (1992)). This type of analogue peptide provides extremely rapid tumor penetration and

even distribution throughout tumor mass compared to IgG or Ig fragments Fab and F(ab')$_2$.

Bifunctional scF$_v$-Fxn or Fxn-scF$_v$

An example of this type of analogue peptide is a V$_L$-linker-V$_H$ with an
5 effector peptide such as a hormone, enzyme, transmitter, and the like. These
hybrid analogue peptides may be prepared as described by McCarney et. al.
(McCarney, J.E. et al., "Biosynthetic Antibody Binding Sites: Development of a
Single-Chain F$_v$ Model Based on Antidinitrophenol IgA Myeloma MOPC 315", J.
Protein Chem. (US) 10 (6):669-83 (1991)). A bi-functional hybrid analogue
10 peptide containing an F$_c$-binding fragment B of staph protein A amino terminal
to a single-chain analogue F$_v$ region of the present specificity is also
encompassed and may be prepared as previously described. (Tai, M.S., et al.,
Biochem. 29 (35):8024-30 (1990)). In this example of a hybrid analogue peptide
of this invention is a Staph. A fragment B (anti F$_c$)) - scF$_v$ polypeptide. The order
15 is backward of normal cases. This FB-sF$_v$ may be encoded in a single synthetic
gene and expressed as peptide in E. coli. This analogue peptide is a good
example of a useful multifunctional targetable single-chain polypeptide. A hybrid
analogue peptide also comprising antibodies to a human carcinoma receptor and
angiogenin is also part of this invention. Angiogenin is a human homologue of
20 pancreatic RNAse. This is an (Fab')$_2$-like antibody-enzyme peptide effector.
Another hybrid analogue peptide comprising a V$_H$-CH1 heavy chain-RNAse may
be expressed in a cell that secretes a chimeric light chain of the same antibody.
A secreted antibody of similar structure was shown to cause the inhibition of
growth and of protein synthesis of K562 cells that express the human transferrin
25 receptor (Rybak, S.M., et al., "Humanization of Immunotoxins", PNAS 89:3165-
3169 (1992)).

Bi-specific Antibodies

A monoclonal antibody or antibody fragment may be incorporated into
a bi-specific analogue peptide as described, for example, by Greenman et al.
30 (Greenman, J., et al., Mol. Immunol. (England) 28 (11):1243-54 (1991). In this
example, a bi-specific F(ab')$_2$ comprising two (Fab'-(thioether-link)-Fab') was
constructed. Bi-specific antibodies may also be obtained when two whole
antibodies are attached. Another way to obtain bi-specific antibodies is by
mixing chains from different antibodies or fragments thereof. In this manner the
35 "left" branch of the bi-specific antibody has one function while the "right" branch
has another.

Phage Display Libraries

The analogue peptides in accordance with this invention may be screened with a filamentous phage system. This system may also be used for expressing any genes of antibodies or fragments thereof as well as for screening for

5 mutagenized antibody variants as described by Marks et al. (Marks, J.D., et al., "Molecular Evolution of Proteins on Filamentous Phage. Mimicking the Strategy of the Immune System", J.Mol. Biol. (England) 267 (23):1607-10 (1992)). A library of V_H and V_x genes or analogue thereof may be cloned and displayed on the surface of a phage. Antibody fragments binding specifically to several

10 antigens may be isolated as reported by Marks (Marks, J.D., "By-Passing Immunization. Human Antibodies from V-gene Libraries Displayed on Phage", J. Mol. Biol. (England) 222 (3):581-97 (1991)).

Covalent Oligosaccharide Modifications

The present analogue peptides alone or as hybrid peptides comprising

15 antibodies and fragments thereof may be, e.g., covalently modified utilizing oxidized oligosaccharide moieties. The hybrid analogue peptides may be modified at the oligosaccharide residue with either a peptide labeled with a radioisotope   such   as ^{125}I or   with   a   chelate   such   as   a diethylenetriaminepentaacetic   acid   chelate   with ^{111}In.   The   use   of

20 oligosaccharides provides a more efficient localization to a target than that obtained with antibodies radiolabeled either at the amino acid chain lysines or tyrosines (Rodwell, J.D. et al., "Site-Specific Covalent Modification of Monoclonal Antibodies: In Vitro and In Vivo Evaluations", PNAS (USA) 83:2632-6 (1986)).

25 Of the analogue peptides of this invention, preferred are those having the sequences ID Nos. 67 through 73, 75 through 81, 95, 96, and analogues thereof wherein about 1 to 42 amino acids in the FR are substituted per chain with amino acids such as those present in equivalent positions in antibodies of the species for which the analogue is intended, such as human, or fragments thereof

30 comprising 1 to 3 CDRs per chain and flanking regions thereof, each of about 1 to 10 or more amino acids, alone or with an N-terminal fragment of about 1 to 10 amino acids, or up to the complete N-terminal region, or combinations thereof. Examples of possible substitute amino acids in all positions, including the most important positions are shown in Tables 10 and 11 above, and are

35 indicated in the columns titled BrE-3 and KC-4, "Most Preferred Analogue and Preferred Analogue" amino acids substituents. Others are also suitable as may be deduced by the method described herein. These amino acid sequences may be bound by a peptide or non-peptide linker such as is known in the art.

Examples of peptide linkers are polylysines, leucine zippers, EGKSSGSGSEJKVD,

and (GGGGS)x3, and non-peptide polymers, among others. Effector agents such

as peptides and non-peptides may also be attached to the analogue peptides of

the invention.   These include non-peptide polymers, monomers, atoms, etc.,

5   which are discussed below.

Another preferred embodiment comprises a bi-functional analogue peptide

having a pair of light and heavy chains of the same specificity attached to one

another by a linker, such as those provided above.   In another preferred

embodiment, a bi-functional analogue peptide comprises one set of light and

10   heavy chains comprising at least one xenogeneic CDR or variable region, e.g.,

the amino acid sequence ID No. 11 or 13 of Tables 15 and 16, sequence ID No.

95 or 96 from Tables 55 and 56 below, or the respective CDRs alone or

separated by 1 to 10 amino acids and up to the entire amino acid segments of

their flanking sequences, and optionally with 1 to 10 amino acids of each N-

15   terminal region, sequence ID No. 68, 70 and/or 72, alone or with flanking

sequences of varying length, or 76, 78 and/or 80, alone or with flanking

sequences of varying length, with the modifications shown above, wherein about

1 to 42 amino acids in the FR are substituted per chain with amino acids such

as those present in equivalent positions in antibodies of the target species, or

20   fragments thereof comprising 1 to 3 CDRs per chain and flanking regions thereof,

each of about 1 to 10 or more amino acids, alone or with an N-terminal fragment

of about 1 to 10 or more amino acids, and one set of light and heavy chains

comprising at least one xenogeneic CDR or analogue variable region, e.g., amino

acid sequence ID No.11, 13, 95, or 96 having a different set of substitute amino

25   acids, wherein about 1 to 46 or more amino acids in the FR are substituted per

chain with amino acids such as those present in equivalent positions in

antibodies of the target species, or fragments comprising 1 to 3 CDRs per chain

and flanking regions thereof, each of about 1 to 10 or more amino acids, alone

or with an N-terminal fragment of about 1 to 10 or more amino acids, or

30   fragments or combination thereof. Multi-functional hybrid analogue peptides may

comprise several identical units or combinations of the above bi-functional

analogue peptides of the same or different specificities or xenogeneic species.

Preferred analogue peptides are those comprising murine CDRs and other regions

substituted with human amino acids.

35   In another aspect, this invention provides a hybrid analogue polymer that

comprises at least one anti-tumor analogue peptide and at least one effector

agent operatively linked to the peptide, combinations thereof and mixtures

thereof. The effector agent utilized in this invention comprises peptide polymers

other than the constant region of an antibody of the same species as the CDRs,

HuKC4V3, ATCC No. HB 11456 deposited on September 23, 1993, all of which deposited under the Budapest Treaty as an example of a best mode of this invention).

     The hybrid analogue polymer may comprise two heavy and two light

5   chains, each light and heavy chain comprising at least one CDR or analogue variable region polypeptide or fragments thereof of one species and the constant region and the substitute amino acids of an antibody of a different species such as human, at least one other CDR, analogue variable region, chimeric Fab , Fab' or (Fab')$_2$, fragments thereof, combinations thereof, and mixtures thereof. Still

10   more preferred is a hybrid analogue peptide comprising at least two "humanized" murine-human or chimeric antibody fragments thereof, Fab, Fab' or (Fab')$_2$ fragments thereof operatively linked to one another. The peptide fragments may be covalently attached to one another as is known in the art (Marchis-Mouren G., et al., "HT 29, a Model Cell Line: Stimulation by the Vasoactive Intestinal Peptide

15   (VIP); VIP Receptor Structure and Metabolism", Bioch. 70 (5):663-71 (1988)), or they may be synthesized by methods known in the art (Allen, G., et al., "Production of Epidermal Growth Factor in Escherichia Coli from a Synthetic Gene", J. Cell Sci. Suppl. 3:29-38 (1985)).

     The hybrid analogue polymer of the invention described above may have

20   two heavy and two light analogue chains operatively linked to one another, where each pair of heavy and light chains, has specificity for a different epitope. One example of this analogue peptide is a pair of "humanized" variable region heavy and light chains of a BrE-3 analogue peptide and a pair of "humanized" variable region light and heavy chains of a KC-4 analogue peptide that are

25   covalently attached to one another by a peptide or non-peptide polymer or a disulfide bridge, or non-covalently by means of a leucine zipper or two helical structures, and the like. Non-peptide polymers may be covalently attached to peptides by methods known in the art (Duronio, V., et al., "Two Polypeptides Identified by Interleukin 3 Cross-Linking Represent Distinct Components of the

30   Interleukin 3 Receptor", Exp. Hematol. 20 (4):505-11 (1992)). In another embodiment, the invention provides a hybrid analogue peptide comprising at least one CDR or analogue variable region of the heavy chain of an antibody of a first species or fragments thereof, operatively linked to a first effector agent, and at least one CDR or analogue variable region of the light chain of an antibody

35   of a second species or fragments thereof operatively linked to a second effector agent and combinations thereof, wherein each pair of light and heavy chains has a predetermined specificity, combinations thereof, and mixtures thereof. In another preferred embodiment of the hybrid analogue peptide, at least one CDR or analogue variable region of the heavy chain of a murine antibody or fragments

thereof and at least one CDR or variable region of the light chain of a murine antibody or fragments thereof are linked to one another by a non-peptide polymer such as an isoprene polymer or monomer. In still another preferred embodiment, the hybrid analogue peptide of the invention is one wherein at least one pair of

5   light and heavy chains comprising at least one murine CDR or analogue variable region or fragment thereof is linked to at least one other pair of light and heavy chains comprising at least one murine CDR or analogue variable region or fragment thereof. In another embodiment the two or more F_v regions are covalently attached to one another by a peptide or non-peptide polymer or a

10 disulfide bridge, or non-covalently by means of a leucine zipper or two helical structures, and the like. In a most preferred embodiment, the analogue peptides and hybrid polypeptides of the invention have affinity and specificity for an epitope located in the most hydrophilic region of a 20 amino acid tandem repeat that makes up a large part of the polypeptide core of mammary mucin, to

15 hexamer fragments of the sequence APDTRPAPG or trimer TRP fragments shown to afford the strong binding of all five different monoclonal antibodies raised against the human mammary fat globule (Mc1, Mc5, BrE-1, BrE-2 and BrE-3). The monoclonal antibodies were shown to bind to different but overlapping polypeptide epitopes but to have different tissue and tumor specificities, to

20 quantitatively differ in their binding to tumor cells such as breast carcinoma cell lines when observed by flow cytometry and have different competition patterns for binding to the native antigen on breast carcinoma cells. Also preferred amongst antibodies utilized for the preparation of the present analogue peptide and hybrid polypeptide are those that exhibit strong binding to the hexamer

25 peptides described above or to fragments comprising a TRP trimer to tandem repeats thereof. In one most preferred embodiment, the analogue peptide comprises the humanized mutein antibody expressed by the hybridoma cell line having the ATCC Accession No. HB 11200 (BrE-3 HZ). This cell was deposited with the ATCC under the Budapest Treaty on November 13, 1992. In another

30 most preferred embodiment, the analogue peptide comprises the humanized mutein antibody expressed by the hybridoma cell line having the ATCC Accession No. HB 11455 (HuKC-4V2), deposited under the Budapest Treaty on September 23, 1993. These were deposited as the best mode of the invention known to the inventors.

35   The anti-tumor analogue peptide and/or hybrid analogue polymer of the invention are also provided as an anti-tumor composition along with a carrier or diluent, preferably a pharmaceutically-acceptable carrier or diluent. The anti-tumor analogue peptide and the hybrid polymer provided herein may be present in the composition in an amount of about 0.001 to 99.99 wt%, more preferably

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about 0.01 to 20 wt%, and still more preferably about 1 to 5 wt%. However, other amounts are also suitable. Carriers generally, and pharmaceutically-acceptable carriers in particular are known in the art and need not be further described herein. The carrier may be provided in a separate sterile container or

5   in admixture with the polypeptide. Typically, saline, aqueous alcoholic solutions, albumin-saline solutions, and propylene glycol solutions are suitable. However, others may also be utilized. When utilized for therapeutic purposes the proteic material must be of a purity suitable for human administration, and the composition may contain other ingredients as is known in the art. Examples of

10   these are other anti-neoplastic drugs such as adriamycin and mitomycin, cytoxan, PALA and/or methotrexate, among others. However, other therapeutic drugs, carriers or diluents, immunological adjuvants and the like may be also be added. When the composition described above is utilized for in vivo imaging, it may comprise about 0.001 to 99.9 wt% analogue peptide, and more preferably

15   about 0.01 to 25 wt% analogue peptide. Typically, when the composition is utilized for therapeutic purposes it may contain about 0.001 to 99.9 wt% analogue peptide, and more preferably about 0.01 to 30 wt% analogue peptide. When utilized for the ex vivo purging of neoplastic cells from bodily fluids such as spinal fluid, the composition may comprise about 0.0001 to 50wt%, and

20   preferably about 0.01 to 20wt% analogue peptide. When applied to the in vitro diagnosis of tumors such as carcinomas the composition of the invention may comprise about 0.001 to 35 wt% analogue peptide, and more preferably about 0.01 to 10 wt% analogue peptide. Other amounts, however, are also suitable.

Such products find one utility in the treatment of tumors such as

25   carcinomas, such as breast, lung, ovary, endometrial, pancreas, prostate and colon cancers, among others. The "humanized", "half humanized" and "partially humanized" analogue peptides may be used for the in vivo treatment or diagnosis of humans. The "animalized", "half animalized" and "partially animalized" analogue peptides of the invention may be utilized for the treatment of non-

30   human species such as were described above insofar as the amino acids of the species are substituted for those of the xenogeneic amino acids present at a specific location, and any constant region present in the analogue. The present analogue peptides are particularly suitable for repeated administration to a subject and for long term therapy, such as is the case of metastases and/or the

35   reoccurrence of tumors. Of all analogues described and encompassed herein, the ones most suitable for in vivo applications are those that exhibit low or no binding to serum antigens and to normal cells. Suitable for in vitro or ex vivo uses are those that exhibit good binding to tumor cell antigens such as the carcinoma cell antigen and weak or no binding to normal cells. Even though a

patient may have in circulation an interfering amount of a molecule that can bind the analogue peptide, the peptide may still be administered after removal of such serum molecule either by ex-vivo procedures or by administration of flush doses of the analogue peptide or fragments thereof.

5   A kit for the diagnosis of tumors such as carcinomas may comprise a composition comprising the anti-tumor analogue peptide of the invention, a solid support, anti-tumor antibody (positive control), immunoglobulins of a different species selectively binding the constant regions of the antibody, protein G or protein A, and instructions for its use. This diagnostic kit may be utilized by

10   covalently attaching the antigen or the analogue peptide of the invention or a fusion protein thereof to the solid support by means of a linker as is known in the art. In a particularly preferred embodiment, the support is coated with a polypeptide such as methylated albumin as described in US Patent No. 4,572,901, the relevant text of which is incorporated herein by reference. When

15   a biological sample is added to a well, the analogue peptide of the invention will bind any tumor antigen, such as a carcinoma antigen, present in the biological sample. If a competitive assay is utilized, to the solid supported antigen or hybrid peptide thereof are added a known amount of the analogue peptide and the sample. Thereafter, γ-globulin, protein G or protein A in labeled form may

20   be added for detection. Anti-tumor antibodies of a first species may be obtained by challenging a subject of another species with tumor cells such as carcinoma cells, the human milk fat globule mucin and the like, as is known in the art (Peterson, J.A., et al., Hybridoma 9:221 (1990); US Patent No. 4,708,930). Monoclonal antibodies may be prepared as described by Kohler and Milstein

25   (Kohler, G. and Milstein, C., "Continuous Culture of Fused Cell Secreting Antibody of Predefined Specificity", Nature 256:495-497 (1975)). Suitable for use in this invention are antibodies such as IgG, IgM, IgE, IgA, and IgD. Protein A, protein G and γ-globulin may be obtained commercially.

       A diagnostic kit for detecting tumors such as carcinomas, and more

30   particularly human carcinomas is provided herein that comprises an anti-tumor composition comprising a hybrid analogue peptide and an effector agent comprising an enzyme, a radioisotope, a fluorescent label and/or a peptide comprising the constant region of an antibody of the species for which use it is intended, or fragments thereof capable of binding anti-constant region

35   immunoglobulins, protein G or A, anti-tumor antibody, anti-constant region immunoglobulins, protein G or protein A, a solid support having operatively linked thereto an antigen which specifically binds to the anti-tumor hybrid analogue peptide of the invention and the antibody, and instructions for its use. When the effector agent comprises a peptide, such as the constant region of an antibody

of the target species, the solid support may have operatively linked thereto an antibody which specifically binds to a portion of a fusion protein other than the antigen of the invention. This permits the binding of the anti-tumor analogue peptide to the antigen molecule now attached to the solid support. Any complex

5 formed between the hybrid analogue peptide of the invention and the supported tumor antigen will, thus, remain attached to the solid substrate. A competitive assay may then be conducted by addition to the solid supported antigen of a known amount of the hybrid antigen peptide and the sample. The amount of antigen present in the sample may be obtained from a dilution curve by addition

10 of anti-constant region immunoglobulins, protein G, protein A or other antibody binding molecules, e.g., labeled, to bind the hybrid analogue peptide that is now attached to the support. This kit may be used in a competitive assay where the supported antigen molecule competes with antigen in the sample for a known amount of the analogue peptide of the invention. The assay was described by

15 Ceriani, R.L., et al. (Ceriani, R.L., et al., Anal. Biochem. 201:178-184 (1992)), the relevant text thereof being incorporated herein by reference.

A tumor such as a carcinoma may be imaged in vivo and/or diagnosed by administering to a subject suspected of carrying a carcinoma the anti-tumor analogue peptide of the invention in radiolabeled form, in an amount effective to

20 reach the tumor cells and bind thereto, and detecting any localized binding of the labeled analogue peptide to the tumor. Typically, the analogue peptide of the invention may be administered in an amount of about 0.001 to 5000 mg/kg weight per treatment, more preferably about 0.01 to 5000 μg/kg weight per treatment, and more preferably about 0.1 to 500 μg/kg weight per treatment.

25 However, other amounts may also be utilized. Radiolabels that may be utilized are ^{111}In, ^{125}I, ^{99m}Tc, and ^{131}I, among others. These radioisotopes may be detected with a PET scanner, NMR imaging, and radioactivity counting apparatus that are in wide use by the medical community.

The presence of a tumor such as a carcinoma may also be diagnosed in

30 vitro by contacting a biological sample with the anti-tumor analogue peptide or hybrid polypeptide of the invention to form an anti-tumor analogue peptide-antigen complex with any tumor antigen present in the sample, and detecting any complex formed. The biological sample is typically obtained from a subject such as a human suspected of being afflicted with the tumor. Suitable biological

35 samples are serum, blood, sputum, feces, lymph fluid, spinal fluid, lung secretions, and urine, among others. Clearly, any source of fluid, tissue and the like may be prepared for use in this method as is known in the art.

Neither the hybrid half chimeric/half humanized BrE-3 analogue peptide nor the chimeric BrE-3 polypeptide of the invention or the murine BrE-3 antibody

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show substantially strong binding to normal tissue. The hybrid BrE-3 analogue peptide shows a binding pattern similar to the chimeric BrE-3 polypeptide and the murine BrE-3 antibody. The murine BrE-3 antibody was shown to bind with specificity to various tumors such as carcinomas of the breast, lung, ovary,

5 bladder, and the endometrium, mesothelioma, colon, kidney, liver, pancreas, salivary glands, sarcomas and thyroid, among others. Weak binding was only shown to normal breast tissue, lung tissue, distal convoluted tubes of the kidney, acini of the pancreas and stomach mucosa (Peterson, J.A., et al. (1990), supra). The KC-4 hybrid murine peptide has tissue specificity similar to that of the

10 murine KC-4 antibody. The KC-4 monoclonal antibody was shown to bind specifically and strongly to solid tumor tissue in the lung, colon, kidney, breast, stomach, prostate, pancreas, lymph node duct and lymphoma, and non-specifically and weakly to normal breast, kidney, and stomach tissue. KC-4 also showed some weak binding to normal tissue including spinal cord, uterus,

15 thyroid, tongue, prostate, spleen, adrenal, lung, gall bladder, heart, lymph nodes, colon, liver, brain, testes, thymus, and placenta (U.S. Patent No. 4,708,930). In one preferred embodiment of the in vitro diagnostic method, the anti-carcinoma analogue peptide added to the biological sample comprises a labeled hybrid analogue peptide. Suitable labeling materials were described above. This

20 method may be practiced, with the solid support containing kit described above, as a competitive assay as disclosed by Ceriani, R.L., et al.(Ceriani, R.L., et al. (1992), supra).

The present analogue peptides are also applicable to the purging of neoplastic cells, such as carcinoma cells, from biological samples, be it fluid or

25 tissue samples. The purging of neoplastic cells from a fluid sample is part of the invention and may be practiced by contacting a biological fluid suspected of comprising neoplastic cells with the analogue peptide of the invention, which is capable of selectively binding to an antigen of the neoplastic cells and allowing the peptide to bind to the antigen, and separating the analogue peptide-cell

30 complex from the remainder of the fluid.

This method may be utilized for purging unwanted cells ex vivo by extracting a biological sample from a patient, eliminating the neoplastic cells therefrom by separation of the analogue peptide-cell complexes or by further addition of an effector such as complement or a toxin or a radioactive label that

35 can act upon the cell and then replenishing the purged sample to the patient. This is typically suitable for use with spinal taps where spinal fluid is rid of neoplastic cells such as carcinoma cells prior to reinjection. Other fluids may also be treated in this manner.

The present analogue peptides may also be applied to the histochemical

assessment of the presence of neoplastic cells such as carcinoma cells in a
tissue obtained from a subject suspected of being afflicted by a carcinoma by
methods that are standard in the art, like the preparation of tissue slices and
fixation on a solid substrate to permit the application of the peptide and then the

5   assessment of any binding to neoplastic cells in the sample as indicated by the
formation of complexes between the analogue peptide and antigens on or in the
cells.

       The growth or the size of a primary or metastasized tumor or neoplasia
such as a carcinoma may be inhibited or reduced by administering to a subject

10   in a need of the treatment an effective amount of the anti-tumor hybrid analogue
peptide of the invention. Typically, the hybrid analogue peptide may be
administered in an amount of about 0.001 to 2000 μg/kg body weight per dose,
and more preferably about 0.01 to 500 mg/kg body weight per dose. Repeated
doses may be administered as prescribed by the treating physician. However,

15   other amounts are also suitable. Generally, the administration of the hybrid
analogue peptide is conducted by infusion so that the amount of radiolabel, toxin
or other effector agent present that may produce a detrimental effect may be
kept under control by varying the rate of administration. Typically, the infusion
of one dose may last a few hours. However, also contemplated herein is the

20   constant infusion of a dose for therapeutic purposes that will permit the
maintenance of a constant level of the hybrid polypeptide in serum. The infusion
of the hybrid analogue peptide of the invention may be conducted as follows.
Intravenous (I.V.) tubing may be pretreated, e.g., with 0.9 % NaCl and 5%
human serum albumin and placed for intravenous administration. The prescribed

25   dose of the analogue peptide may be infused as follows. Unlabeled analogue
peptide may be infused initially. 30 minutes after completion of the unlabeled
antibody infusion, ^{111}In-labeled and ^{90}Y labeled antibody may be co-infused. The
I.V. infusion may comprise a total volume of 250 ml of 0.9 % NaCl and 5 %
human serum albumin and be infused over a period of about 2 hours depending

30   on any rate-dependent side effects observed. Vital signs should be taken every,
e.g., 15 minutes during the infusion and every one hour post infusion until
stable. A thorough cardiopulmonary physical examination may be done prior to,
and at the conclusion, of the infusion. Medications including acetaminophen,
diphenhydramine, epinephrine, and corticosteroids may be kept at hand for

35   treatment of allergic reactions should they occur. The administration of the
hybrid analogue peptide of the invention may be repeated as seen desirable by
a practitioner. Typically, once a first dose has been administered and imaging
indicates that there could be a reduction in the size of the tumor, whether
primary or metastasized, repeated treatments may be administered every about

1 to 100, and more preferably about 2 to 60 days. These repeated treatments
may be continued for a period of up to about 2 years, and in some circumstances
even for longer periods of time or until complete disappearance of the tumor(s).
The administration of the hybrid analogue peptides of this invention is typically

5   more useful for therapeutic purposes when a primary tumor has, for example,
been excised. Thus, it is primarily, for mopping up after surgical intervention or
in cases of cancerous metastases that the present method is of most use.

A pure, isolated analogue polydeoxyribonucleotide that comprises an
analogue oligodeoxyribonucleotide encoding the analogue peptide or hybrid

10   peptide of this invention, including all redundant sequences may be applied to the
preparation of the peptide. In one preferred embodiment, the analogue
polydeoxyribonucleotide of the invention comprises a DNA sequence selected
from the group consisting of DNA sequence ID No. 64 of Table 45, or DNA
sequence ID No. 65 of Table 46, or DNA segments encoding the CDR fragments

15   sequence ID No. 68, 70 and/or 72, or 76, 78 and/or 80 flanked by 1 to 10 or
more amino acids, and the N-terminal fragment of 1 to 10 or more amino acids
of human origin. The above DNA sequences may be cloned for expression under
the same promoter. Similarly preferred are also the DNAs having the sequence
ID No: 93 and/or 94 of Tables 52 and 53 and the segments encoding their CDR

20   fragments alone or separated by DNA segments encoding 1 to 10 or more
flanking amino acids and/or terminated by DNA segments encoding 1 to 10 or
more amino acids of the N-terminus.

Also provided herein is a hybrid vector that comprises a vector having the
analogue polydeoxyribonucleotide of this invention operatively linked thereto.

25   Typically, vectors capable of replication both in eukaryotic and prokaryotic cells
are suitable. When the preparation of a glycosylated analogue polypeptide is
desired the vector should be suitable for transfection of eukaryotic host cells.
In one preferred embodiment, the hybrid vector comprises the analogue
polydeoxyribonucleotide and a polydeoxyribonucleotide comprising an

30   oligodeoxyribonucleotide encoding an effector peptide, the effector peptide-
encoding polydeoxyribonucleotide being operatively linked to the vector. As
already indicated, the various DNA sequences may be cloned for expression
under the same promoter. In addition, the polydeoxyribonucleotide encoding the
effector polypeptide may also be cloned for expression under the same promoter.

35   This invention also encompasses a host cell that has been transfected
with the hybrid vector described above. Suitable hosts are prokaryotic and
eukaryotic hosts such as bacteria, yeast, and mammalian cells such as insect
cells and non-producing hybridoma cells, among thers. Suitable vectors and/or
plasmids for the transfection of each one of these types of hosts are known in

the art and need not be further described herein. Also known in the art are methods for cloning DNA sequences into each one of these types of vectors and for transfecting the different types of host cells. Particularly preferred is the cell line having the ATCC Accession No. HB 11200 (BrE-3 HZ).

5 Polyribonucleotides may be obtained by transcription of the polydeoxyribonucloetides described above as is known in the art. Provided herein are analogue polyribonucleotides comprising analogue oligoribonucleotides encoding at least one CDR or an analogue variable region or fragments thereof, combinations thereof, and combinations thereof with an effector peptide may be

10 prepared by cloning the desired DNA segments and then transcribing the thus obtained hybrid polydeoxyribonucleotide into the corresponding RNA sequences.

The analogue peptide which specifically binds to an antigen on the surface or in the cytoplasm of a neoplastic cell, or is released by the cell, may be produced by a method that comprises cloning the analogue

15 polydeoxyribonucleotide of the invention into a vector to form a hybrid vector, transfecting a host cell with the hybrid vector and allowing the expression of the anti-tumor analogue peptide, and isolating the anti-tumor polypeptide or mixtures thereof. The DNA segment encoding the analogue polypeptide may be obtained by chemical synthesis or by site-specific modification of the sequence encoding

20 the variable region of the xenogeneic species by PCR amplification with specifically designed primers as is known in the art. The fragment DNAs may also be prepared by PCR with primers that introduce a stop codon at a desired position as is known in the art. Preferably, the cloning and transfection steps are conducted by cloning polydeoxyribonucleotides encoding the analogue peptides

25 selected from the group comprising at least one CDR or analogue variable region of the heavy or light chains of the xenogeneic species, antibodies thereof, or fragments thereof. The method may further comprise allowing the expressed analogue peptides to interact with one another to form double chain analogue peptides, one or both analogue peptide chain comprising at least one xenogeneic

30 CDR or variable region of the light or heavy chain of the antibody or fragment thereof modified as described above. Still part of this invention is a method of producing a hybrid analogue peptide comprising an effector peptide and an analogue peptide which specifically binds to an antigen on the surface or in the cytoplasm of a tumor cell such as a carcinoma cell or that is released by the cell,

35 the method comprising transfecting a host cell with the hybrid vector of this invention carrying a DNA sequence encoding the hybrid analogue peptide, allowing the expression of the hybrid analogue peptide, and isolating the hybrid analogue peptide or mixtures thereof. The techniques for obtaining mRNA, conducting reverse transcription and PCR amplification of DNA, chemical

synthesis of primers, cloning DNA sequences into a vector, transfecting a host cell, and purifying polypeptides from a culture medium are known in the art and need not be further described herein.

Another aspect of this invention relates to an anti-idiotype peptide that
5 comprises polyclonal antibodies raised against anti-tumor antibodies, the analogue peptide of the invention, monoclonal antibodies thereof, fragments thereof selected from the group consisting of CDRs, Fab, Fab', (Fab')$_2$, and variable region fragments and fragments thereof, analogues thereof selected from the group consisting of Fab, Fab', (Fab')$_2$, and variable regions thereof,
10 wherein about 1 to at least 46 amino acids in the FRs are substituted per chain with amino acids selected from the group consisting of amino acids present in equivalent positions in human antibodies, or fragments thereof comprising 1 to 3 CDRs per chain and flanking regions thereof, each of about 1 to 10 or more amino acids, alone or with an N-terminal fragment of about 1 to 10 or more
15 amino acids. The technique for obtaining anti-idiotype polypeptides is known in the art and need not be further described herein (Nisonoff, A. and Lamoyi, "Implication of the Presence of an Internal Image of an Antigen in Anti-Idiotype Antibodies: Possible Applications to Vaccine Production", Clin. Immunol. Immunopathol. 21:397-406 (1981)). Moreover, the technique for producing
20 hybridomas secreting monoclonal antibodies of a certain specificity is also known in the art (Kohler, G. and Milstein, C. (1975), supra). Techniques for obtaining different antibody fragments were described above or are known in the art and need not be further described herein (Wilbanks, T., et al., "Localization of Mammary Tumors In Vivo with ^{131}I-Labeled Fab Fragments of Antibodies Against
25 Murine Mammary Epithelial (MME) Antigens", Cancer 48:1768-1775 (1981)). The techniques for modifying peptides to obtain the analogue peptides of this invention have been described above or are known in the art.

The hybrid anti-idiotype polymer may further comprise an effector agent operatively linked to the anti-idiotype polypeptide. Effector agents suitable for
30 use herein were described above for the anti-tumor analogue polypeptide of the invention are also suitable for use with the anti-idiotype polypeptide. Preferred hybrid polymers are polyclonal antibodies raised against the anti-tumor monoclonal antibodies or the analogue peptide of the invention, and a monoclonal antibody obtained by fusion of a B-cell producing an antibody having
35 specificity for the analogue peptide of the invention and an immortalized cell line. Also preferred are fragments of the monoclonal antibody such as Fab, Fab', (Fab')$_2$ and variable region fragments, analogues and fragments thereof as described above, and CDRs. Also, as described above for the anti-tumor polypeptide analogue, preferred are combinations of the above fragments and

analogues and combinations of the fragments with whole antibodies and analogues thereof. In another preferred embodiment, the anti-idiotype polymer comprises an analogue variable region of a monoclonal antibody linked to a peptide comprising the hexamers or trimers described above or tandem repeats

5   thereof.

DNA and RNA segments encoding the anti-idiotype polymer and hybrid polymer, a hybrid vector having the DNA operatively linked thereto and a host cell transfected with the hybrid vector are also contemplated herein.

Also provided herein is an anti-tumor vaccine that comprises the anti-

10   idiotype polypeptide of the invention, and a pharmaceutically-acceptable carrier. Typically, the anti-idiotype polypeptide is present in the composition in an amount of about 0.001 to 99.99 wt%, and more preferably about 0.01 to 50 wt% of the composition.   However, other amounts are also suitable. Pharmaceutically-acceptable carriers are known in the art and need not be further

15   described herein.   The vaccine provided herein may further comprise other ingredients such as adjuvants, and the like. Examples of adjuvants are SAF-1 and Freund's, among others. Suitably, other ingredients typically used for the preparation of vaccines may also be utilized herein. In one embodiment, the vaccine of the invention may be provided in unit form as a powder or in a

20   diluent. In another embodiment, it may be provided in powder form in a sterile container comprising a plurality of doses for preparation prior to utilization. Diluents that are suitable for the preparation of a formulation that may be administered to a patient by injection are known in the art. Examples were provided above.

25   An anti-tumor vaccination kit is also provided by this invention that comprises, the vaccine described above and a diluent   in separate sterile containers, and instructions for its use.

An effective amount of the anti-idiotype polypeptide or hybrid polypeptide described above may be administered to vaccinate a human. Typical

30   amounts administered to vaccinate a human are about 0.001 to 5000 mg/kg body weight/dose, more preferably about 0.1 to 5000 μg/kg body weight/dose. The anti-idiotype vaccine of the invention may be administered repeatedly in order to boost the active immunization produced by the first dose. An anti-idiotype antibody very likely resembles the epitope on the neoplastic cell to

35   which the anti-tumor antibody binds. Thus, it may be utilized for the production of an immunological response by a subject such as a human or other mammals against its own neoplastic cells.

When an anti-idiotype polypeptide of, e.g., non-human origin is administered to a, e.g., human, it may produce a somewhat detrimental

response. Accordingly, in theory, the smaller the non-human amino acid sequence the anti-idiotype polypeptide contains, the lesser the immunogenic response to its xenogeneic sequences it will elicit in a human. Accordingly, preferred anti-idiotype polypeptides are those containing at least one CDR or

5 variable region of a non-human antibody binding specifically to the anti-tumor polypeptide described herein, optionally as a hybrid polypeptide. Also preferred are human anti-idiotype antibodies, CDR and variable fragments thereof, and fragments thereof that are operatively linked to an effector agent comprising a human polypeptide that may include the constant region of a human antibody

10 and fragments thereof, non-peptide polymers, monomers and atoms that may be radiolabeled as described above. Other types of constructs are also possible, several of which were described above.

Peptides comprising the sequence APDTRPAPG or fragments thereof comprising hexamers with the trimer TRP or TRP by itself or tandem

15 repeats thereof may also be utilized for the preparation of the fusion protein, particularly as part of the antigenic peptide. The peptide comprising the hexapeptide or tripeptide sequences may be utilized as a tandem repeat comprising up to about 10,000 repeats of the basic unit, an in some instances up to about 500,000 repeats. In another embodiment, peptides comprising one

20 or more hexapeptides or tripeptides may be operatively linked to other polypeptide sequences of related or unrelated function, which sequences provide bulk that aids the clearance through the liver and/or kidneys of the immunological complex formed between the circulating unbound or residual antibody or polypeptides utilized for the therapy of neoplasias such as carcinomas and the

25 hexapeptide. The peptides comprising the hexapeptide or tripeptide may also be provided as a hybrid analogue peptide with other analogue peptides described above. In the absence of such treatment, the therapeutic antibody, which may carry a radioisotope, a toxin or other therapeutic molecules, may remain in the circulation for several days and in some instances weeks. This, in the case of a

30 radioactively labeled antibody or analogue of the invention may produce extensive damage, which is highly detrimental to the health of the patient, and in some instances lethal.

Thus, the serum concentration of a circulating antibody or polypeptide that binds to an antigen on the surface or in the cytoplasm of tumor cells such

35 as carcinoma cells or released by the cells may be lowered by administering to a subject the anti-idiotype polypeptide described above, in an amount effective to bind the circulating polypeptide, to thereby accelerate its clearance. Typically, the anti-idiotype polypeptide or hybrid polymer are administered in an amount of about 0.001 to 5000.00 mg/kg body weight/dose, more preferably about 0.01

5000 mg/kg body weight/dose. However, other amounts may also be utilized. he administration of the anti-idiotype polypeptide may be infusion as described ove.

The growth or the size of a primary or metastasized tumor may be hibited or reduced by administering to a subject in need of the treatment an ffective amount of an antibody or an anti-tumor hybrid analogue peptide omprising an effector agent selected from the group consisting of radioisotopes, herapeutic drugs and vaccines, and an anti-tumor polypeptide which specifically inds to an antigen on the surface or in the cytoplasm of a tumor cell or ɔleased by the cell, allowing the hybrid polypeptide to reach the tumor and the olypeptide to bind thereto, and administering to the subject an amount of the nti-idiotype polypeptide of the invention effective to bind residual or unbound irculating hybrid analogue peptide to thereby accelerate the clearance of the ybrid polypeptide.

Having now generally described this invention, the same will be better nderstood by reference to certain specific examples, which are included herein ɔr purposes of illustration only and are not intended to be limiting of the ɪvention or any embodiment thereof, unless so specified.

EXAMPLES

Example 1: Methods Utilized

The procedures utilized herein for the reverse-transcription (RT) of RNAs ɛncoding the variable regions and the subsequent amplification of the cDNAs by the polymerase chain reaction (PCR) have been described (Orlandi, R., et al., "Cloning Immunoglobulin Variable Domains for Expression by the Polymerase Chain Reaction", PNAS (USA) 86:3833-3837 (1989); Coloma, M.J., et al., "Primer Design for the Cloning of Immunoglobulin Heavy-Chain Leader-Fvs from Murine Hybridoma Cells Using the PCR", Bio.Techniques 11:152-156 (1991); Gavilondo-Cowley, J.V., et al., "Specific Amplification of Rearranged Immunoglobulin Fv Genes from Murine Hybridoma Cells", Hybridoma 9:407-417 (1990)).

Total RNA is an adequate substrate for RT-PCR. Polyadenylated RNA was utilized herein, however, because it contains only minor levels of contaminating ribosomal RNA and practically no DNA. The polyadenylated RNA was isolated with a Fast Track mRNA isolation kit (Invitrogen Corporation, San Diego, CA).

The oligonucleotides were synthesized on a PCR-Mate EP DNA synthesizer model 391 (Applied Biosystems, Foster City, CA). A PCR murine Ig

ɪnplementary DNA

ɪs, Norwalk, CT).

000, using a TA ƆNA was isolated ι sequencing was ɑtes Biochemical, ɪmol (Amersham

ɪnputer using the

) were cultured ɪne serum (FBS), ɪ) or RPMI:FBS, ɑcterial growth. supplement as The freezing

, MuIgV$_H$5'-C, ɪrchased from r primers were ble 12 below.

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Example 4:   Cloning of Chimeric BrE-3 Antibody
              Polydeoxyribonucleotide

Two expression vectors pAG4622 and pAH4604 were utilized herein
(Coloma, M.J., et al., "Novel Vectors for the Expression of Antibody Molecules
5   Using Variable Regions Generated by PCR", J. Immunol. Methods 152:89-104
(1992)). These were kindly provided by S.L. Morrison (Dept. of Microbiology
and Molecular Genetics, UCLA). The construction and expression of chimeric
genes was performed as described by Coloma et al. (Coloma, M.J., et al. (1992),
supra).

10   Oligonucleotides were synthesized and used in a PCR mixture to produce
variable heavy (V_H) and variable light (V_L) fragments with the correct ends for
insertion into the pAG4622 and pAH4604 expression vectors. There sequences
are shown in Table 13 below.

Table 13: Synthesized Oligonucleotides

15

JO16 (senseV_H leader) - GGG GATATC CACC ATG TAC TTG GGA CTG AAC TAT GTC TTC A (Seq. ID No: 6);
JO17 (sense V_L leader) - GGG GATATC CACC ATG AAG TTG CCT GTT AGG CTG TTG GT (Seq. ID No: 7);
JO18 (anti-sense JH3) - GGG GCTAGC TGC AGA GAC AGT GAC CAG AGT CC   (Seq. ID No: 8);
JO19 (anti-sense Jκ1) - GGG GTCGACTTAC G TTT GAT TTC CAG CTT GGT   GCC TCC A (Seq. ID No: 9).

The original pCR1000 clones were utilized as the starting templates for
25   the PCR. The new PCR products were cloned back into pCR1000 and their
sequence confirmed. Correctly modified and amplified fragments were excised
with either EcoR V and Sal I (for V_L) or with EcoR V and Nhe I (for V_H). These
fragments were then ligated into the respective vectors, which had been cut
open with the appropriate restriction enzymes. Both the vectors and the inserts
30   were purified from an agarose gel prior to ligation, using the Bio101 GeneClean
kit (glass beads) (La Jolla, CA).

Example 5:   Expression of Murine-Human
              Chimeric BrE-3 Antibody

The V_H and V_L regions in the final murine-human chimeric antibody were
35   sequenced once again to verify that their sequences were correct.

The non-producer myeloma cell line SP2/0-Ag14, (ATCC: CRL 1581,
Shulman, M., et al., "A Better Cell Line for Making Hybridomas Secreting
Specific Antibodies", Nature 276:269-270, (1978)) was transfected, and a
chimeric antibody were isolated as described by Coloma, M.J. et al. (1992), with
40   the following modification. The selection was only undertaken for the uptake of
hisD by adding 5mM histidinol to the medium and readjusting the pH to 7.4 with

NaOH.

Example 6: Production of Transfected Hosts

     After ten days, the stable transfectant colonies were clearly established at a frequency of approximately 10^{-5}. The colonies were transferred to a normal

5   medium (without histidinol) and the supernatants from stable transfectants were assayed for the presence of the murine-human chimeric BrE-3 antibody. This was done by capturing the secreted murine-human chimeric BrE-3 antibody with a plate-bound goat anti-human-κ antibody and developing with goat anti-human-γ antibody as described by Coloma et al. with the following modification. The

10 secondary antibody utilized herein was radiolabeled with ^{125}I.

Example 7:   Confirmation of Murine-Human
                Chimeric BrE-3 Antibody Expression

     The supernatants were assayed for binding to human milk fat globule (HMFG) as described by Ceriani et al. (Ceriani R.L., et al., Diagnostic Ability of

15 Different Human Milk Fat Globule Antigens in Breast Cancer", Breast Cancer Res. Treat. 15:161-174 (1990)). HMFG was bound to the microtiter plates as described previously (Ceriani R.L., "Solid Phase Identification and Molecular Weight Determination of Cell Membrane Antigens with Monoclonal Antibodies", in: Monoclonal antibodies and functional cell lines. Progress and application,

20 Bechtol, K.B., McKern, T.J., and Kennett, R., Eds., Plenum Press, New York, pp 398-402 (1984)).

     Most colony supernatants were positive by both assays. The colonies that secreted the highest level of chimeric antibody into the supernatants, as determined by these assays, were subcloned and subsequently adapted to

25 serum-free medium for the purification of antibody.

Example 8:   Determination of Affinity Constants

     The antibody-antigen affinity constants for the murine-human chimeric antibody anti-human milk mucin and the whole murine antibody were determined by obtaining the reciprocal value of the concentration of competing unlabeled

30 monoclonal antibody giving 50% binding as described by Sheldon et al. (Sheldon, K.,et al., "Characterization of Binding of Four Monoclonal Antibodies to the Human Ovarian Adenocarcinoma Cell Line HEY", Biochem. Cell Biol., 65: 423-428, (1987)). The protocol for the assay was as follows.

     Microtiter plates (Dynatech, Chantilly, VA) were prepared   using

35 successive layers of methylated BSA, glutaraldehyde, anti-β-galactosidase and the bacterial fusion protein 11-2 (a hybrid of β-galactosidase and human mammary mucin) as described in Ceriani et al. (Ceriani, R.L., et al., "A Novel

49

Serum Assay for Breast Cancer Epithelial Antigen Using a Fusion Protein", Anal. Biochem. 201:178-184 (1992). Each well contained 388ng of the 11-2 fusion protein. To each well were added 25μl ^{125}I -BrE-3 (ATCC No. HB 10028) in RIA buffer (10% bovine calf serum, 0.3% triton X-100, 0.05% sodium azide pH7.4,

5   in phosphate buffered saline), and competed with 25 μl of either unlabeled murine antibody or murine-human chimeric antibody in RIA buffer at final concentrations in the nanomolar range.

Iodinations were performed with ^{125}I (17 Ci/mg, Nordion International Inc., Kanata, Ontario, Canada). 50 micrograms of monoclonal antibody BrE-3

10   (Coulter, Hialeah, FL) were labeled at a specific activity of 9.56 mCi/mg using the chloramine T method as described by Ceriani, R.L. and Blank, E.W., (Ceriani, R.L., and Blank, E.W., "Experimental Therapy of Human Breast Tumors with 131I-Labeled Monoclonal Antibodies Prepared Against the Human Milk Fat Globule", Cancer Res. 48:4664-4672 (1988)). When the counts of bound

15   radiolabeled anti-BrE-3 murine antibody were plotted an the Y axis and the logarithm of the nanomolar (nM) concentration of competing unlabeled anti-BrE-3 murine antibody or murine-human chimeric antibody were plotted in the X axis, both curves overlapped within 5% error (Figure not shown). This proves that the variable region's affinity characteristics have been preserved.

20   **Example 9:**   Affinity Binding Constants for BrE-3 Murine
                  and Murine-Human Chimeric Antibody

The purified murine-human chimeric BrE-3 antibody and purified murine BrE-3 antibody gave similar competition curves when tested against ^{125}I-labeled murine BrE-3 binding to its antigen. The affinity binding constants of the murine

25   antibody and the murine-human chimeric antibody were determined in independent competition assays as described in Example 8 above. The values of the constants are 2.68×10^{6} M^{-1} and 3.75×10^{6} M^{-1} for the hybrid chimeric BrE-3 polypeptides and for the murine BrE-3 antibody, respectively. These values are not distinguishable at a 95% confidence interval.

30   **Example 10:**   Amplification of cDNAs Encoding
                  BrE-3 Antibody Variable Regions

The cDNAs that encode the anti-BrE-3 murine immunoglobulin variable domains (V$_H$ and V$_L$) were prepared by reverse transcription and PCR amplification (RT-PCR) from polyadenylated RNA isolated from 10^{6} BrE-3

35   hybridoma cells by the following procedure. The JO2, JO3, JO4, JO14 and V$_H$1BACK primers were synthesized, and their sequences shown in Example 3 above. Other primers were purchased from Novagen. With the exception of V$_H$1BACK, which is a framework-specific primer, all sense primers are specific

for the leader peptide region. All anti-sense primers are specific for the constant

regions. The degenerate λ chain of the specific primer MulgλV$_L$3'-1 (from

Novagen), was used to isolate the κ chain cDNA clones because of the similarity

of the λ and κ. An identical κ chain clone was isolated with primer JO2 which

5 is specific for the κ chain constant domain. The V$_H$ region cDNA could not be

isolated with the available leader peptide primers. Thus, the V$_H$1BACK primer

was used, which yielded the V$_H$ cDNA γ72. The leader-peptide primer JO14

was then designed by extrapolating from the framework sequence of γ72, using

cataloged nucleotide sequences (Kabat, E.A., et al., "Sequences of Proteins of

10 Immunological Interest", U.S. Dept. Health and Human Services, NIH publication

No. 91-3242, 5th Edition (1991). After sequential PCR reactions, this new primer

yielded the complete V$_H$ framework cDNA. This information is summarized in

Table 14 below.

Table 14: Primer Combinations for PCR Amplification

15

	Clone No.	Sense Primers	Antisense Primers
V$_L$	152	MulgκVL5'-C	JO2
	164	MulgκVL5'-C	MulgλVL3'-1
V$_H$	γ72	V$_H$1BACK	(JO3 or JO4)
	1012	JO14(1st PCR)	JO3
		JO14(2nd PCR)	JO4
	1043	JO14(1st PCR)	JO3
		(MulgVH5'-C +	MulgγVH3'-2
		MulgVH5'-F)	
		(2nd PCR)	

Example 11:   Isolation of Amplified BrE-3 V$_L$
              and V$_H$ cDNA and Sequences

30   The PCR products were cloned without prior purification into pCR1000

(Invitrogen) and sequenced in both directions. Clones 152, 164, 1012, and

1043 were isolated independently during different RT-PCR runs. The sequences

of V$_L$ clones 152 and 164 were found to be identical, as were the sequences of

the V$_H$ clones 1012, 1043. The V$_H$ and V$_L$ DNA sequences and their derived

35 protein sequences are shown in Tables 15 and 16 below.

Table 15: BrE-3 V$_L$ Nucleotide and Derived Protein Sequences

DNA Sequence

ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG TTG TTC TGG ATT CCT GCT TCC ATC AGT GAT GTT GTG ATG ACC CAA ACT CCA CTC TCC CTG

CCT GTC AGT CTT GGA GAT CAA GCT TCC ATC TCT TGC AGA TCT AGT CAG AAC CTT GTA CAC AAC AAT GGA AAC ACC TAT TTA TAT TGG TTC

CTG CAG AAG TCA GGC CAG TCT CCA AAG CTC CTG ATT TAT AGG GCT TCC ATC CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA

TCA GAG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TTT CAA GGT ACA CAT GTT CCG_TGG

ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA C (Seq. ID No: 10)

Amino Acid Sequence

m k l p v r l l v l L F W I P A S I S D1 V V M T Q T P L S L

P V S L G D Q A S I S C R S S Q N L V H N N G N T Y L Y W F

L Q K S G Q S P K L L I Y R A S I R F S G V P D R F S G S G

S E T D F T L K I S R V E A E D L G V Y F C F Q G T H V P W

I F G G G T K L E I K (Seq. No: 11)

Table 16: BrE-3 V$_H$ Nucleotide and Derived Protein Sequences

DNA Sequence

ATG TAC TTG GGA CTG AAC TAT GTC TTC ATA GTT TTT CTC TTA AAA GGT GTC CAG AGT GAA GTG AAG CTT GAG GAG TCT GGA GGA GGC TTG

GTG CAA CCT GGA GGA TCC ATG AAA CTC TCT TGT GCT GCT TCT GGA TTC ACT TTT AGT GAT GCC TGG ATG GAC TGG GTC CGC CAG TCT CCA

GAG AAG GGG CTT GAG TGG GTT GCT GAA ATT AGA AAC AAA GCC AAT AAT CAT GCA ACA TAT TAT GAT GAG TCT GTG AAA GGG AGG TTCACC

ATC TCA AGA GAT GAT TCC AAA AGT AGA GTG TAC CTG CAA ATG ATA AGC TTA AGA GCT GAA GAC ACT GGC CTT TAT TAC TGT ACT GGG GAG

TTT GCT AAC TGG GGC CAG GGG ACT CTG GTC ACT GTC TCT GCA G (Seq. ID No: 12)

Amino Acid Sequence

m y l g l n y v f I V F L L K G V Q S E1 V K L E E S G G G L

V Q P G G S M K L S C A A S G F T F S D A W M D W V R Q S P

E K G L E W V A E I R N K A N N H A T Y Y D E S V K G R F T

I S R D D S K S R V Y L Q M I S L R A E D T G L Y Y C T G E

F A N W G Q G T L V T V S A (Seq. ID No: 13)

The sequences were interpreted as described by Kabat et al. (1991). The residues that are shown in lower case correspond to PCR primers. The mature chains begin at D1 (V$_L$) and E1 (V$_H$), respectively. The amino-acids that are underlined are those corresponding to the CDRs. The underlined nucleotides

5 indicate joining segments.

The framework and CDR polypeptide segments were identified according to Kabat et al. (1991), supra. V$_L$ is a group IIκ chain. Part of the CDR 3 and all of framework 4 (FR4) are encoded by J$_{\kappa1}$. V$_H$ belongs to group IIIc. CDR 3 and FR4 are encoded by J$_{H3}$. Little or nothing remains from an unidentified D minigene.

Thus, the CDR 3 is only 4 amino-acids long.

Example 12:   Comparison of cDNA deduced Amino Acid
                Sequence with Directly Determined
                N-Terminal Fragment Sequence

5   Table 17 below shows a comparison between the cDNA-derived polypeptide

sequence and the polypeptide sequence determined directly from purified murine

BrE-3 monoclonal antibody.

Table 17: Comparison of cDNA-Deduced Protein Sequence with
           Directly Determined N-terminal Protein Sequence

VL	cDNA-deduced	DVVMTQTPLSLPVSLGDQASISCRS
VL	Protein sequence	GVVMTQTPLSLPVVLGDQASIIXRX
VH	cDNA-deduced	EVKLEESGGGLVQPGGSMKLSCAAS
VH	Protein sequence	EVKLEESGGVLVQPGGSMKLSSAAS

The murine BrE-3 antibody was reduced with 5% mercaptoethanol,

separated on a 10% SDS polyacrylamide gel, and electroblotted onto a ProBlott

membrane (Applied Biosystems, Foster City, CA). Amino acid sequencing was

20   performed directly on the immobilized bands by the Biotechnology

Instrumentation Facility, University of California, Riverside. The protein

sequence given here is the sequencer's best guess.

Once the variable region cDNAs were cloned, it was confirmed that, in fact,

they encoded the variable regions of the murine BrE-3 antibody and not those of

25   another antibody by comparing the cDNA-derived amino acid sequences of the

cloned murine BrE-3 antibody variable region with the N-terminal sequence of the

purified anti-BrE-3 murine antibody directly determined by a single run of protein

sequencing. The cDNA sequences were shown to be accurate by comparison

with two independently reverse transcribed clones.

30   The general agreement between the predicted and the determined

amino-acid sequences shows that the cloned cDNAs encode polypeptides of the

same class and subclass as the variable regions of the murine BrE-3 antibody.

This indicates that the cDNAs encode authentic variable regions. The

authenticity of the variable region polypetide and, therefore, that of the murine-

35   human chimeric BrE-3 antibody is unquestionable given that the variable regions

and the chimeric antibody affinity constant are indistinguishable from those of

the murine BrE-3 antibody.

Example 13:   Construction of Murine-Human
               Chimeric Antibody Genes

The vectors used were developed by Coloma et al. (Coloma, M.J., et al.
(1992), supra) and kindly provided by S.L. Morrison (Dept. of Microbiology and
5   Molecular Genetics, UCLA). Both vectors were derived from pSV2 (Mulligan,
R.C., and Berg, P., "Expression of a Bacterial Gene in Mammalian Cells", Science
209:1422-1427 (1980)), and contain genomic fragments encoding either the
heavy or the light chain constant domains. The vectors accept cDNAs that
encode the F_v regions. To ligate the F_v cDNAs to the vectors, restriction ends
10   were added to the cDNAs in a set of PCR reactions, using the JO16, JO17,
JO18 and JO19 primers.

The pAG4622 light chain vector contains the gene for the human κ chain
constant region, including the J-C intron. It encodes xanthine-guanine
phosphoribosyltransferase or gpt (Mulligan, R.C., and Berg, P., "Selection for
15   Animal Cells that Express the Escherichia Coli Gene Coding for Xanthine-Guanine
Phosphoribosyltransferase", PNAS (USA) 78:2072-2076 (1981)) as a dominant
selectable marker. It accepts the murine VL cDNA between the ribosome
binding site (Kozak, M., "Compilation and Analysis of Sequences Upstream from
the Translational Start Site in Eukaryotic mRNAs", Nucleic Acids Res.
20   12:857-872 (1984)), which is preceded by the VH promoter from the anti-dansyl
murine monoclonal antibody 27.44 (Coloma, M.J., (1992), supra), and the J-C
intron.   The J-C intron contains the k chain enhancer (Potter, H., et al.,
"Enhancer-Dependent Expression of Human κ Immunoglobulin Genes Introduced
into Murine Prep-B Lymphocytes by Electroporation", PNAS (USA)
25   81:7161-7165 (1984); Emorine, L., et al., "A Conserved Sequence in the
Immunoglobulin J Kappa-C Kappa Intron: Possible Enhancer Element", Nature
304: 447-449 (1983)).

The pAH4604 heavy chain vector contains the gene for the heavy chain γ1
constant region, but no J-C intron. It encodes histidinol-dehydrogenase or hisD
30   (Hartman, S.C. and Mulligan, R.C. Two Dominant-Acting Selectable Markers for
Gene Transfer Studies in Mammalian Cells", PNAS (USA) 85:8047-8051 (1988))
as a dominant selectable marker. It accepts the murine V_H cDNA between the
dansyl promoter-ribosome binding site and the constant γ1 gene. The vector
also contains an insert that encodes the heavy chain enhancer (Rabbitts, T.H.,
35   et al, "Transcription Enhancer Identified Near the Human C mu Immunoglobulin
Heavy Chain Gene is Unavailable to the Translocated c-myc Gene in a Burkitt
Lymphoma", Nature 306:806-809 (1983)).

The new V_H and V_L DNA fragments with appropriate restriction ends were
integrated into pAH4604 and pAG4622 as described in Example 4 above. The

vectors were then electroporated (together) into SP2/0-Ag14 myeloma cells as described by Coloma et al. (1992), supra.

Example 14:   Characterization of Murine-Human
                Chimeric BrE-3 Antibody

5   The supernatants from stable transfectants were assayed for the presence of the murine-human chimeric antibody as described in Examples 6 and 7 above. High producing transfectants were subcloned and subsequently adapted to grow in serum-free medium. The murine-human chimeric antibody produced by the myeloma cell line was then purified from the culture supernatant using a

10   Sepharose 4B-protein A column (Bio-Rad, Richmond, CA) as described in Ey, P.L., et al. (Ey, P.L., et al., "Isolation of Pure IgG1, IgG2a and IgG2b Immunoglobulins from Murine Serum Using Protein A-Sepharose", Immunochemistry 15:429-436 (1978)). Antibody disulfide bonds were reduced to separate the light and heavy chains by heating for 10 min at 65° in Laemmli

15   loading buffer containing 5% beta-mercaptoethanol. The separated chains were then chromatographed on a SDS polyacrylamide gel (10%). The reduced murine-human chimeric BrE-3 antibody and murine antibodies were eletrophoresed in separate lanes next to 97.4, 66.2, 45.0, 31.0 and 2.5 Kdalton protein markers. Table 18 below shows the apparent molecular weights of the two bands

20   obtained for both.

Table 18:   Apparent Molecular Weights of Light and Heavy
               Chains of Murine and Chimeric BrE-3 Antibodies

Chimeric Antibody		Murine Antibody	
Heavy Chain (Kd)	Light Chain (Kd)	Heavy Chain (Kd)	Light Chain (Kd)
50	30	49	29

The heavy and light chains of the anti-BrE-3 chimeric antibody separate as expected when electrophoresed on a polyacrylamide gel.

Example 15: Tissue Binding Studies with Chimeric BrE-3 Antibody

30   Immunohistochemical staining using the immunoperoxidase technique of consecutive human breast carcinoma tissue sections was conducted with the murine-human chimeric BrE-3 antibody. A control was stained with the anti-human secondary antibody only. Positive staining resulted from the use of the murine-human chimeric BrE-3 antibody, followed by the anti-human antibody

35   specific binding. (Pictures not shown).

The breast carcinoma tissue sections were stained with the supernatant

of the transfected cells using the Vectastain ABC method (Vector Labs, Burlingame, CA). The tissue stained with the goat anti-human Ig secondary antibody only shows background or non-specific staining of necrotic areas of the tissue section.

5   The tissue stained with murine-human chimeric BrE-3 antibody, followed by the secondary antibody, shows specific staining of the breast carcinoma cells in the breast tissue sections.

Example 16: Chimeric BrE-3 Antibody Imaging Studies

        The anti-BrE-3 murine monoclonal antibody has been shown to be highly

10   effective for imaging and for the radioimmunotherapy of breast cancers. For example, in a pharmacokinetic study of 15 breast cancer patients conducted with an ^{111}In-MXDTPA-BrE-3 radio-immunoconjugate (anti-BrE-3 antibody), the serum levels were low in most patients, the blood clearance correlated with the circulating antigen and the imaging results showed that about 86% of all sites

15   could be imaged (Liebes, L., et al., "Pharmacokinetics of ^{111}In-BrE-3 Monoclonal Antibody in Patients with Breast Carcinoma", Proc. Am. Assoc. Cancer Res. 33:216(Abs No. 1292) (1992)). A ^{90}Y-BrE-3 radioimmunoconjugate having similar pharmacokinetic characteristics and extrapolating the ^{111}In-BrE-3 dosimetry results provide a superior therapeutic agent, as well.

20   As with many other monoclonal antibodies, however, the clinical applications of the anti-BrE-3 murine antibody, a whole murine antibody, are limited by the HAMA response. A chimeric monoclonal antibody should give a more restricted HAMA response.

Example 17: Chimeric BrE-3 Antibody Immunogenicity

25   The BrE-3 variable region murine polypeptides have been cloned without the constant regions to produce less immunogenic polypeptides than the parent murine antibody. It has, moreover, been shown herein that the murine-human chimeric BrE-3 antibody lacking its original murine constant region preserves its antigen binding characteristics.

30   An BrE-3 antibody variable region alone or as a murine-human chimeric antibody also containing a constant human region or fragment thereof would be significantly less immunogenic to humans than the parent murine antibody. The hybrid polypeptide comprising the variable region of the BrE-3 antibody and the constant region of a human antibody was shown to preserve the original binding

35   affinity of the murine antibody. In this hybrid polypeptide, approximately 2/3 of its contiguous non-human immunogenic targets (C_L and C_H regions) were entirely replaced by human constant domains.

Example 18: PCR Primers used in First Isolation of Anti-KC-4 cDNAs

The PCR primers were purchased from Novagen (Madison, WI). Their

sequences, reproduced from the booklet provided by Novagen, are shown in

5　Table 19 below.

Table 19: PCR Primer Sequences

MuIgκV$_L$5'-C: sense primer mix for kappa leader.
ACTAGTCGACATGAAGTTGCCTGTTAGGCTGTTGGTGCTG (Seq. ID No: 14)
10　ACTAGTCGACATGGAGWCAGACACACTCCTGYTATGGGT(Seq. ID No: 15)
ACTAGTCGACATGGATTTWCAGGTGCAGATTWTCAGCTTC (Seq. ID No: 16)

MuIgκV$_L$3'-1: antisense kappa constant region.
CCCAAGCTTACTGGATGGTGGGAAGATGGA (Seq. ID No: 17)

15　MuIgV$_H$5'-F: sense primer mix for heavy chain leader.
ACTAGTCGACATGRACTTTGGGYTCAGCTTGRTTT (Seq. ID No: 18)
ACTAGTCGACATGAGAGTGCTGATTCTTTTGTG (Seq. ID No: 19)
ACTAGTCGACATGGATTTTGGGCTGATTTTTTTTATTG (Seq. ID No: 20)

MuIgγV$_H$3'-2: antisense gamma constant region.
20　CCCAAGCTTCCAGGGRCCARKGGATARACIGRTGG (Seq. ID No: 21)

Example 19:　Cloning of Murine-Human Chimeric
anti-KC-4 Antibody Ribonucleotide
The two expression vectors pAG4622 and pAH4604 described in

25　Example 4 were utilized. Oligonucleotides synthesized and used in a PCR to

produce V$_H$ and V$_L$ fragments with the correct ends for insertion into the

pAG4622 and pAH4604 expression vectors are shown in Table 20 below.

Table 20: PCR Primers Sequences

30　JO20 - sense kappa leader
GGG GATATC CACC ATG AAG TTG CCT GTT AGG CTG TTG (Seq. ID No: 22)

JO21 - antisense JK2
CCC GTCGACTTAC G TTT TAT TTC CAG CTT GGT CCC CCC T (Seq. ID No: 23)

JO22 - sense V$_H$ leader
35　GGG GATATC CACC ATG GAC TTT GGG CTC AGC TTG GTT TT (Seq.ID No: 24)

JO24 - antisense JH3
CCC GCTAGC TGC AGA GAC AGA GAC CAG AGT CC (Seq. ID No: 25)

The original pCR1000 clones were the starting templates for the PCR and the

40　rest of the procedures as described in Example 4 above.

<u>Example 20:</u> Expression of the anti-KC-4 Chimeric Antibody Gene

15   The V$_H$ and V$_L$ regions in the anti-KC-4 murine-human chimeric antibody were sequenced once again to verify that their sequences were correct. The transfection of the non-producer myeloma cell line SP2/0-Ag14, (ATCC: CRL 1581) and isolation of polypeptide was conducted as described in Example 5 above.

20   <u>Example 21:</u> Production of Transfected Hosts

          After ten days, stable transfectant colonies were clearly established at a frequency of approximately 1/10,000. The colonies were transferred to normal medium and the assays conducted as described in Example 6 above.

<u>Example 22:</u>   Confirmation of anti-KC-4 Murine-
25   Human Chimeric Antibody Expression

          The supernatants were assayed for binding to human milk fat globule (HMFG) and the breast epithelial mucin (BEM) as described previously in Example 7 above. HMFG and BEM were bound to the microtiter plates as described previously by Ceriani, R.L. (1990), supra. In this radioassay the bound anti-KC-4 30   chimeric antibody (HMFG and BEM) was detected by anti-human gamma chain conjugated to ^{125}I. Most colony supernatants were positive by both assays. The colonies that secreted the highest level of chimeric antibody in the supernatants, as determined by these assays, were subcloned.

<u>Example 23:</u> Western Blot

35   75 μl of the culture supernatant was added to 20 μl of 4x Laemmli buffer and 5 μl β-mercaptoethanol and the mixture was heated at 65 ºC for 15 min., in order to reduce antibody disulfide bonds and, thus, separate heavy from light chains. 20 μl of the treated sample was chromatographed in duplicate lanes on a 10% SDS polyacrylamide gel together with other antibodies that were treated 40   similarly and that were loaded for comparison. Pre-stained size markers (BioRad, Richmond, CA) were also loaded.

5   The chromatographed proteins were electroblotted onto a ProBlott membrane (Applied Biosystems, Foster City, CA) in 90% 30 mM CAPS pH11, 10% methanol, for 1 hour at 25 V and at 4°C. The membrane was cut into 2 parts containing identical antibody samples. The 2 membranes were immersed in 20% bovine calf serum in PBS and shaken slowly at room temperature for 10   1 hour 35 min. ^{125}I-labeled goat anti-human κ chain antibody was added to one membrane and ^{125}I labeled goat anti-human γ chain antibody to the other

membrane. Antibodies were labeled at a specific activity of approximately 10 mCi/mg using the chloramine T method as described by Ceriani, R.L. and Blank, E.W. (1988), the labeled antibodies were diluted to 4,000 cpm/μl in RIA buffer.

After incubating 3 hours at room temperature the blots were washed
5   twice in TBS for 10 min each time, once in TBST (50 mM TRIS pH7.5, 3 mM EDTA 25 mM NaCl) 10 min and once more in TBS (TBS with 0.05% Tween 20) for 10 min. The membranes were dried and exposed to Kodak XAR film.

Western blot analysis of culture supernatants revealed that three antibody chains were expressed that corresponded to the three antibody chains
10   seen in the original anti-KC-4 murine antibody. These were a heavy chain that stained with goat anti-human γ chain ^{125}I-labeled antibody, and two light chains that stained with goat anti-human κ chain ^{125}I-labeled antibody (Figure not shown).

The treatment of the original anti-KC-4 murine antibody with
15   N-glycosidase F (Boehringer Mannheim GmbH Germany) following the recommendations of the manufacturer, produced a noticeable decrease in the intensity of the "top" light chain and a concomitant increase in the intensity of the bottom light chain (Figure not shown).

The explanation for the existence of an extra light chain is that this chain
20   is glycosylated. Three lines of evidence substantiate this. First, the detection of an asparagine-linked glycosylation site in the amino acid sequence of the light chain. That is the triad NIS (Asn-Ile-Ser) in framework 3. Second, the decrease of the intensity in the putative glycosylated band after treatment with N-glycosidase F, while concomitantly the intensity of the non-glycosylated band
25   was increased. Finally, 2 corresponding light chain bands are seen in the chimeric antibody version. The extra light chain in the chimeric version cannot be a contaminant since it was specifically stained by goat anti-human κ chain antibody. It can only be a product expressed by pAG4622. Thus both light chains must have the same V_L amino acid sequence and the same human
30   constant region. These observations show that approximately half of the light chains of both the anti-KC-4 murine and chimeric antibodies are glycosylated at the asparagine-linked glycosylation site.

Example 24:   Amplification of cDNAs Encoding
                   anti-KC-4 Antibody F_v Regions

35   The cDNAs that encode the anti-KC-4 murine immunoglobulin V_H and V_L were prepared as described in Example 9 above from polyadenylated RNA isolated from 100 million KC-4 hybridoma cells. All clones were obtained from independent PCRs. The sequences of the primers are given in Example 19 and

20 above. All primers are specific for either the leader peptide region or for the constant regions. The primer combinations utilized herein are shown in Table 21 below.

Table 21: Primer Combination for PCR Amplifications

	Clone No.	Primer combinations
V_L	96	MulgκV$_L$5'-C + MulgκV$_L$3'-1
	107	MulgκV$_L$5'-C + MulgκV$_L$3'-1
	K1	JO20 + JO21
V_H	66	MulgV$_H$5'-F + MulgγV$_H$3'-2
	209	MulgV$_H$5'-F + MulgγV$_H$3'-2
	H3	JO22 + JO24
	H7	JO22 + JO24

Example 25:   Isolation of Amplified anti-KC-4 F_{VL} (V_L)
and F_{VH} (V_H) cDNA and Sequences

The PCR products were cloned, without prior purification, into pCR1000 (Invitrogen) and sequenced in both directions. The V_H and V_L DNA sequences and their derived protein sequences are shown in Tables 22 and 23 below.

Table 22: V_L Nucleotide Sequences
anti-KC-4 V_L (kII-Jk2)

```
ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG ATG TTC TGG ATT CCT
GCT TCC AGC AGT GAT GTT TTG ATG ACC CAA ACT CCT CTC TCC CTG
CCT GTC AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT
CAG AGC ATT GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC
CTG CAG AAA CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT
TCC ATC CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA
TCA GGG ACA GAT TTC ACA CTC AAT ATC AGC AGA GTG GAG GCT GAG
GAT CTG GGA ATT TAT TAC TGC TTT CAA GGT TCA CAT GTT CCG TAC
ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA C (Seq. ID No: 26)
```

Table 23: V_H Nucleotide Sequences
anti-KC-4 V_H (IIID-D9-JH

```
ATG GAC TTT GGG CTC AGC TTG GTT TTC CTT GTC CTT ATT TTA AAA
GGT GTC CAG TGT GAA GTG CAG ATG GTG GAG TCT GGG GGA GTG AAG
CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC GCT
TTC AGT AGC TAT GCC ATG TCT TGG GTT CGC CAG GAG AAG AGG CTG
GAG TGG GTC GCA GAA ATT AGT AGT GGT GGT AAT TAC GCC TAC TAT
CAA GAC ACT GTG ACG GGC CGA TTC ACC AGA GAC AAT GCC AAG AAC
ACC CTG TAC CTG GAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC
ATG TAT TAC TGT GCA AGG GAG GGT ATC CCG GCC TGG TTT GCT TAC
TGG GGC CAA GGG ACT CTG GTC TCT GTC TCT GCA G (Seq. ID No: 27)
```

Example 26: Amino Acid Sequences of anti-KC-4
Chimeric Antibody Fv Regions

5   After the anti-KC-4 F_v region cDNAS were cloned, and sequenced, and their cDNA-derived amino acid sequence was compared with the N-terminus sequence directly determined by a single run of amino acid sequencing on purified anti-KC-4 antibody. The cDNA sequences were shown to be accurate since in both cases they were identical for clones that were prepared from

10 independent reverse transcription reactions. This confirms that the cloned cDNAs are authentic anti-KC-4 F_v regions. The sequences are shown in Tables 24 and 25 below.

Table 24: V_L Amino Acid Sequences
anti-KC-4V_L(kII-Jk2)

15

	MKLPVRLLVLMFWIPASSS (Seq. ID No: 28)
FR1	DVLMTQTPLSLPVSLGDQASISC (Seq. ID No: 29)
CDR1	RSSQSIVHSNGNTYLE (Seq. ID No: 30)
FR2	WYLQKPGQSPKLLIY (Seq. ID No: 31)
CDR2	KVSIRFS (Seq. ID No: 32)
FR3	GVPDRFSGSGSGTDFTLNISRVEAEDLGIYYC (Seq. ID No: 33)
CDR3	FQGSHVPYT (Seq. ID No:34)
FR4	FGGGTKLEIK (Seq. ID No. 35)

Table 25: V_H Amino Acid Sequences anti-KC-4V_H (IIID-D9-JH3)

	MDFGLSLVFLVLILKGVQC (Seq. ID No: 36)
FR1	EVQMVESGGGLVKPGGSLKLSCAASGFAFS (Seq. ID No:37)
CDR1	SYAMS (Seq. ID No: 38)
FR2	WVRQSPEKRLEWVA (Seq. ID No: 39)
CDR2	EISSGGNYAYYQDTVTG (Seq. ID No: 40)
FR3	RFTISRDNAKNTLYLEMSSLRSEDTAMYYCAR (Seq. ID No: 41)
CDR3	EGIPAWFAY (Seq. ID No: 42)
FR4	WGQGTLVSVSA (Seq. ID No: 43)

The sequences were interpreted as described by Kabat et al. (1991), supra. The residues that are underlined correspond to PCR primers. The mature V_L and

35 V_H chains begin at amino-acids D and E of framework 1 (FR1), respectively.
        Framework and CDR protein segments were identified according to Kabat et al. (1991), supra. V_L is a group II κ chain. Part of the CDR 3 and all of the

framework 4 (FR4) are encoded by Jk2. V$_H$ belongs to group IIId. CDR 3 and FR4 resulted from a genomic recombination involving minigenes D9 and JH3. There is an asparagine glycosylation site in the light chain in FR3. The site reads NIS (Asn Ile Ser).

5 Example 27:   Comparison of cDNA-deduced Amino
                   Acid Sequence with Directly Determined
                   N-Terminal Fragment Sequence

A comparison between the cDNA-derived polypeptide sequence and the amino acid sequence determined directly on the purified anti-KC-4 monoclonal 10 antibody was undertaken. The results are shown in Table 26 below.

Table 26:   Comparison of cDNA-deduced with Directly
            Determined N-Terminal Amino Acid Sequences

	FIRST BAND TOP
V$_H$, cDNA-deduced	EVQMVESGGGLVKPGGSLKLS (Seq. ID No: 44)
V$_H$, Protein sequence	EVQMVESGGGLVKPGGXLKLS (Seq. ID No: 45)
	SECOND BAND
V$_L$, cDNA-deduced	DVLMTQTPLSLPVSLGDQASI (Seq. ID No: 46)
V$_L$, Protein sequence	DVLMTQTPLSLPVXXGDQASI (Seq. ID No: 47)
	THIRD BAND
V$_L$, cDNA-deduced	DVLMTQTPLSLPVSLGDQASI (Seq. ID No: 48)
V$_L$, Protein sequence	DVLMTQTPLSLPVSLGDQASI (Seq. ID No: 49)

X uncertain or alternative calls.

A sample of anti-KC-4 chimeric antibody (approximately 190 μg) was reduced with 5% beta-mercaptoethanol (65°C for 15 min.), separated on three lanes of a 10% SDS polyacrylamide gel, and electroblotted onto a ProBlott membrane (Applied Biosystems, Foster City, CA) in 90% 30 mM CAPS pH11. 5 10% methanol, for 1 hour at 25 V and at 4°C. The transferred protein species were stained with Commassie Brilliant Blue. 3 bands were seen in each lane, of which 2 migrated as expected for a heavy and light chain. The third band migrated above the light chain. Amino acid sequencing was performed directly on the immobilized bands by the Biotechnology Instrumentation Facility, 10 University of California, Riverside. The amino acid sequence given here is the sequencer's best guess.

<u>Example 28:</u>   Construction of anti-KC-4 Murine-
                      Human Chimeric Antibody Genes

The vector used were described in Example 1 above. Restriction ends
were added to the cDNAs in a set of PCR reactions, using primers J020, 21,
5   22, and 24.

The pAG4622 light chain vector and the pAH4604 heavy chain vector
were described in Example 12 above.

The new V_H and V_L DNA fragments with appropriate restriction ends
were integrated into pAH4604 and pAG4622 as described in Example 12 above.
10   The vectors were then electroporated (together) also as described in Example 12
above.

<u>Example 29:</u>   Tissue Binding Studies

The supernatants from stable transfectants were assayed for the
15   presence of the anti-KC-4 murine-human chimeric antibody as described in
Example 13 above. The chimeric antibody secreted in the supernatant bound
both HMFG and BEM very strongly. In addition, the supernatants containing
anti-KC-4 murine-human chimeric antibody were used to stain human breast
carcinoma tissue sections by using the immunoperoxidase immunohistochemical
20   staining technique. The intensity of the staining was comparable to that
obtained with the original murine monoclonal antibody. The anti-KC-4
monoclonal antibody is known to bind the human milk fat globule and the breast
epithelial mucin. This binding specificity of the anti-KC-4 murine monoclonal
antibody was maintained even after the recombinant procedure. The anti-KC-4
25   chimeric antibody bound very strongly to HMFG and BEM as determined by a
radioassay (Ceriani, et al., Breast Cancer Res. Trent. 15:161 (1990)). In
addition, the anti-KC-4 chimeric antibody bound several human breast tumors in
histopathological sections in a manner comparable to the anti-KC-4 murine
monoclonal antibody, as detected by immunostaining described in Example 15
30   above. This specificity of binding demonstrated the retained binding reactivity
of the variable regions of anti-KC-4 murine antibody by the polypeptide of the
invention when attached to the human F_c fragment.

<u>Example 30:</u>   Materials and Assays for Epitope Mapping

The specific details of the preparation of materials, cell lines, and
35   techniques employed were disclosed by Peterson et al. (Peterson, J.A., et al.,
"Molecular Analysis of Epitope Heterogeneity of the Breast Mucin", Breast
Epithelial Antigens, Ed. Ceriani, R.L., Plenum Press, NY (1991)), the relevant text
of which is incorporated herein by reference.

Overlapping peptide hexamers were synthesized onto the ends of polyethylene pins using an Epitope Scanning Kit (Cambridge Research Biochemicals, Cambridge, UK), which is based on a method originally described by Geysen et al. (Geysen, H.L., et al., "Use of Peptide Synthesis to Probe Vital

5   Antigens for Epitopes to a Resolution of a Single Amino Acid", PNAS (USA) 81:3998-4002 (1984)). The polyethylene pins were arranged in a 8x12 configuration that fits into a 96 well microtiter dish. The pins are supplied with an alanine attached to the ends to which the amino acids are added consecutively using pentafluorophenyl active esters of fluorophenyl-

10   methyloxycarbonyl (Fmoc)-L-amino acids. Each consecutive overlapping hexamer differs from the previous one by a single amino acid and enough were synthesized to span the entire sequence of the peptide to be tested so that every combination of hexamer was present. Each monoclonal antibody was tested for binding to the synthetic peptides using an ELISA method with horseradish

15   peroxidase-conjugated goat anti-murine IgG (Promega, Madison, WI) and color development with 2,2-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid (Sigma, St. Louis, MO).

20   The hexapeptides starting with A,P, D, and T bind well to the antibodies (Hexamers 1 to 3 and 20), whereas the hexamers starting between these positions did not. The hexamers that were prepared are shown in Table 27 below. The linear amino acid sequence essential for its binding to the antigen may be deduced from the hexamer that each monoclonal antibody binds. For

25   example, the anti-BrE-3 antibody required the sequence TRP within the hexamer. Other monoclonal antibodies required other amino acid sequences (e.g., anti-Mc5, TRPAP; anti-Mc1, DTR; anti-BrE-1, DTRP). BrE-2 also required TRP but its different specificity for normal and tumor tissue indicates that its epitope on the native antigen is different from BrE-3.

Table 27: Epitope Mapping of Repeat Peptide Breast Mucin

```
Hexamer   GVTSA     PDTRPAPGSTAPPAHGVTS      APDTRP
 1                  PDTRPA
 2                   DTRPAP
 3                    TRPAPG
 4                     RPAPGS
 5                      PAPGST
 6                       APGSTA
 7                        PGSTAP
 8                         GSTAPP
 9                          STAPPA
10                           TAPPAH
11                            APPAHG
12                             PPAHGV
13                              PAHGVT
14                               AHGVTS
15                                HGVTSA
16                                 GVTSAP
17                                  VTSAPD
18                                   TSAPDT
19                                    SAPDTR
20                                     APDTRP
```

Example 31: Epitope Mapping

Five different monoclonal antibodies (Mc1, Mc5, BrE1, BrE2 and BrE3) were prepared using HMFG for immunization. All identified epitopes on the highly glycosylated large molecular weight breast mucin. By immunohistochemistry they appeared to recognize different epitopes since each had different tissue and tumor specificities (Peterson, J.A., et al., "Biochemical and Histological Characterization of Antigens Preferentially Expressed on the Surface and Cytoplasm of Breast Carcinomas Cells Identified by Monoclonal Antibodies Against the Human Milk Fat Globule", Hybridoma 9:221-235 (1990)). Each monoclonal antibody bound to a different spectrum of normal tissues and their specificities for different carcinomas were different. Anti-BrE2 and anti-BrE3, however, were quite similar. In addition, by screening breast λgtII cDNA expression libraries with some of these monoclonal antibodies, cDNA clones were isolated that produced fusion proteins that bound all of them, while other cDNA clones bound just some (Larroca, D., et al., "High Level Expression in E. Coli of an Alternate Reading Frame of pS2 mRNA that Encodes a Mimotope of Human Breast Epithelial Mucin Tandem Repeat" Hybridoma 11(2):191-201 (1992)). This binding to the fusion proteins indicated that the epitopes for these five monoclonal antibodies included the polypeptide portion of this glycoprotein. To confirm this the binding of these monoclonal antibodies to two synthetic polypeptide 20-mers (PDTRPAPGSTAPPAHGVTSA and APPAHGVTSAPDTRPAPGST) that spanned the tandem repeat consensus sequence was tested (Gendler, S.J., et al., "Cloning of Partial cDNA Encoding

Differentiation and Tumor-Associated Mucin Glycoproteins Expressed by Human Mammary Epithelium", PNAS (USA) 84:6060-6064 (1987); Siddiqui, J., et al., "Isolation and Sequencing of a cDNA Coding for the Human DF3 Breast Carcinoma-Associated Antigen", PNAS (USA) 85:2320-2323 (1988)).

5   One was started at the beginning of the published 20 amino acid repeat (Gendler, S.J., et al. (1987), supra) unit, and the other was started in the middle. All five monoclonal antibodies bound to both synthetic peptides, as did DF3, a monoclonal antibody against breast carcinoma cells produced by others (Hull, S.R., et al., "Oligosaccharide Differences in the DF3 Sialomucin Antigen from Normal Human Milk and the BT-20 Human Breast Carcinoma Cell Line", Cancer Comm. 1:261-267 (1989)). Three other monoclonal antibodies against other components of the HMFG that do not cross-react with the breast mucin, Mc13, against a 70 KDa glycoprotein, and Mc3 and Mc8, against a 46 KDa glycoprotein do not bind to these synthetic peptides (data not shown) (Ceriani, R.L. , et al., "Characterization of Cell Surface Antigens of Human Mammary Epithelial Cells with Monoclonal Antibodies Prepared Against Human Milk Fat Globule", Somat. Cell Genet. 9:415-427 (1982); Peterson, J.A., et al., "Biochemical and Histological Characterization of Antigens Preferentially Expressed on the Surface and Cytoplasm of Breast Carcinoma Cells Identified by Monoclonal Antibodies Against the Human Milk Fat Globule", Hybridoma 9:221-235 (1990)).

Example 32:   Approach for Humanization of Antibodies .

The present humanization approach is based on Padlan, E.A., "Choosing the Best Framework to Use in the Humanization of an Antibody by CDR-Grafting: Suggestions from 3-D Structural Data", Antibody Engineering 2nd. Annual Conf. San Diego, CA (Dec 16-17, 1991).

The fine specificity may be preserved in a "humanized" antibody only if the CDR structures, their interaction with each other, and their interaction with the rest of the variable domains can be maintained. (Padlan, E.A.(1991), supra). This requires the preservation of residues of the FR amino acids which contact the CDRs, those which are involved in the V_L-V_H contact, and those which are buried and could influence the overall domain structure and the structure of the combining site.

By examination of murine Fab structures, for which atomic coordinates are available, the FR amino acids that are probably "important" in maintaining the structure of the combining site may be determined (Padlan, E.A., 8th International Congress of Immunol., Budapest, Hungary, Abstracts p. 19 (August 2-28, 1992)).

The specificity of an antibody depends on the CDR structures and sometimes, on some of its neighboring residues as well. These CDR structures, in turn, depend on contacts with framework amino acids and on the interaction of the V_L and V_H domains. Thus, to ensure the retention of binding affinity, not only the CDR residues must be preserved, but also those FR residues that contact either the CDRs or their opposite domains, as well as all buried residues, which give shape to the variable domains. The buried amino acids are placed in exactly the same positions in human and in murine frameworks (Padlan, E.A., "A Possible Procedure for Reducing the Immunogenicity of Antibody Variable Domains While Preserving Their Ligand-Binding Properties", Molecular Immunology 28:489-498 (1991)).

This approach was applied to design humanized analogues of the variable regions of the murine antibodies of the invention. The humanization or design of the exemplary analogue peptide provided herein was undertaken as follows. The identification of the residues, which are most probably "important" in preserving the combining site structure, permits the selection of the best human FR sequences to use in the "humanization" of each chimeric antibody of known structure or analogues peptides of the invention. The results of the analysis can be used also to predict which FR amino acids should probably be retained in those cases where no three-dimensional structural data are available.

The present procedure was designed to reduce the immunogenicity of the xenogeneic antibodies by preparation of their chimeric derivatives or fragments thereof while preserving their antigen-binding properties. In general, the antigen binding properties of an antibody are primarily determined by its CDRs. The CDRs of the murine antibody were "grafted" herein onto a human framework. In addition, the FR amino acids in the antibody, that are judged as probably important in maintaining the combining-site structure, may also be retained in the humanized molecule.

Example 33:   Choice of a Murine Model of Known Structure
               for the Humanization of the BrE-3 Antibody

The classification of the V_H and V_L domains of an antibody such as the anti-BrE-3 antibody was done according to Kabat, E.A., et al. (Kabat, E.A., et al., "Sequences of Proteins of Immunological Interest" NIH (1991). The anti-BrE-3 kappa chain V_L domain belongs to group II and the V_H domain belongs to group IIIc. A murine antibody was then found, whose structure had been determined, and whose variable regions belong to the same classes. The anti-fluorescyl murine antibody 4-4-20 shown in Table 1 above (Herron, J.N., et al., "Three-Dimensional Structure of a Fluorescein-Fab Complex Crystallized in 2-Methyl-2,4-

Pentanediol", Proteins, 5:271-280 (1989) fits these requirements since, like BrE-3, it has V_L and V_H domains belonging to groups II and IIIC. Thus, the three-dimensional structures of antibodies BrE-3 and 4-4-20 should be similar, and BrE-3 may be modeled after 4-4-20.

5   Example 34:   Choice of Target Human Framework
                     for Humanization of BrE-3 Antibody

The choice of the target human framework was not based on the similarity of the amino acid sequence of the entire framework, but strictly on the similarity at the residues that were judged to be structurally important according

10   to the 4-4-20 model. That is, only amino acids that could be involved in contacts with CDR of the opposite chain, or amino acids whose side-chains were predicted to be inwardly pointed. The positions of these amino acids are shown in Tables 8 and 9 and also in Tables 2, 3, 4, 5 and 6 above. These positions are as follows.

15   For the light chain variable region framework: 1, 2, 3, 4, 5, 6, 11,
            13, 19, 21, 23, 35, 36, 37, 38, 44, 45, 46, 47, 48, 49, 58, 60,
            61, 62, 69, 71, 73, 75, 78, 82, 86, 88, 98, 102, and 104.
            For the heavy chain variable region framework: 4, 6, 12, 18, 20,
            22, 24, 27, 28, 29, 30, 36, 37, 38, 39, 40, 45, 46, 47, 48, 49,
20   66, 67, 68, 69, 71, 73, 76, 78, 80, 82c, 86, 88, 90, 91, 92, 93,
            94, 103, 107, 109 and 111.

The numbering system is conventionally accepted (Kabat, et al. (1991), supra) and shown in Tables 10 and 11 above. In this case, the consensus sequences of all human F_v regions were selected as the target human framework

25   to minimize the immunogenicity of the product.

First, the sequences of the murine variable chains were aligned with consensus sequences from all known human variable region classes (Herron, J.N., (1989), supra) and the number of differences in the amino-acids that must be retained from the murine were scored. The positions of these amino acids

30   were obtained from those of murine monoclonal antibody 4-4-20, which was chosen to model the anti-BrE-3 antibody as shown in Tables 28 and 29 below.

Table 28: Choice of BrE-3 V$_L$ Target Human Framework

```
                                        BrE-3 V_L
                        CDRI                        CDR2
            •••••• •• •••              ••••   ••••••
BRE3K   DVVMTQTPLSLPVSLGDQASISC RSSQNLVHN-NGNTYLY WFLQKSGQSPKLLIY RASIRFS
HuKi-n  .IQ...S.S..SA.V..RVT.T. .A...S..XXS-ISN..A .YQ..P.KA...... A...SLE.
HuKii-nt .I....S......TP.EP..... .....S.L.SXD.....N .Y...P....Q.... LV.N.A.
HuKiii  EI.L..S.GT.SL.P.ER.TL.. .A..SVSSS.—...A .YQ..P..A.R..... G..S.AT
HuKiv   .I....S.D..A....ER.T.N. K...SVLYSS.NKN..A .YQ..P..P....... W..T.E.

                                                         CDR3
            • •••  •• • • • • •• • • ••          • ••
BRE3K     GVPDRFSGSGSETDFTLKISRVEAEDLGVYFC FQGTHVPW-T FGGGTKLEIK
HuKi-n  ...S.......G.....T..SLQP..FAT.Y. Q.YNSL.EW. ..Q...V...
HuKii-nt ..........G...........V...Y. M.ALQX.RX. ..Q...V...
HuKiii  .I.........G.....T...L.P..FA..Y. Q.YGSS.PL. ..Q...V...
HuKiv   ..........G.....T..SLQ...VA..Y. Q.YYST.-X. ..Q...V...
```

. identity with the murine sequence
* the murine residues that are structurally important.

Table 29: Choice of BrE-3 V$_H$ Target Human Framework

```
                                        BrE-3 V_H
                                        CDRI
            •• • •••• ••••           ••••• •••••
BRE3VH  EVKLEESGGGLVQPGGSMKLSCAASGFTFS DAWMD– WVRQSPEKGLEWVA
HuHI    Q.Q.VQ..AEVKK..A.V.V..K...Y..T SYAIS– ....A.GQ....MG
HuIII   Q.Q.Q...P...K.SQTLS.T.TV..GSV. SYXWSWN .I..P.G.....IG
HuHIII  ..Q.V............LR........... SYA.S– ....A.G......S

             CDR2        •••• • • • • • ••' • •  ••••
BRE3VH  EIRNKANNHATYYDESVKG RFTISRDDSKSRVYLQMISLRAEDTGLYYCTG
HuHI        W.N-PYG.GD.N.AQKFQ. .V..TA.T.T.TA.MELS...S...AV...AR
HuHII   R.YYR.YSGS.X.NP.L.S .V...V.T..NQFS.KLS.VT.A..AV...AR
HuHIII  V.SG.TDGGS...AD.... .......N..NTL....N........AV...AR

             CDR3      • • • •
BRE3VH  EF————————AN WGQGTLVTVSA
HuHI        APGYGSGGGCYRGDYXFDY ..........S
HuHII   ELPGGYXGDDYYYXXGFDV ..........S
HuIIII  GRXGXSLSGXYYYYHYFDY ..........S
```

. identity with the murine sequence
* the murine residues that are structurally important.

Based on these scores, the human frameworks belonging to groups V$_k$II and V$_H$III were chosen to receive the anti-BrE-3 CDRs plus other important amino acids.

Example 35: Identification of BrE-3 Murine-Human Differences

5   The original murine sequences (BrE-3 V$_k$ or V$_H$) were aligned with their closest human (Human KII or HIII) relatives that were chosen after comparing their sequences in Table 28 above. The alignment of these two sequences is shown in Table 30 below. The information in this table is also contained in Table

28 above, but is reproduced here for clarity. The CDRs are not shown, since their sequences were not changed during the humanization process. Thus, Table 30 shows the maximum number of amino acids that can be changed toward the humanization of the anti-BrE-3 antibody, based on the consensus human
5   sequences obtained from the current databases (Kabat, E.A., et al. (1991), supra). If all these positions were to be replaced with the corresponding human amino acids, the corresponding CDR grafted antibody variable regions would be attained.

Table 30: Corresponding Amino Acid Sequences of VK BrE-3 and Human KII

```
                         CDR1                      CDR2
BrE-3 VK       DVVMTQTPLSLPVSLGDQASISC ─────── WFLQKSGQSPKLLIY ──
Human KII      .I....S......TP.EP..... ─────── .Y...P....Q.... ──
                                    CDR3
BrE-3 VK       GVPDRFSGSGSETDFTLKISRVEAEDLGVYFC─── FGGGTKLEIK
Human KII      ..........G..............V...Y. ─── ..Q...V...
                                    CDR1
BrE-3 VH       EVKLEESGGGLVQPGGSMKLSCAASGFTFS ── WVRQSPEKGLEWVA
Human III      ..Q.V............LR.......... ── ....A.G......S
                                    CDR2
BrE-3 VH       ─────── RFTISRDDSKSRVYLQMISLRAEDTGLYYCTG
Human III      ─────── .......N..NTL....N.......AV...AR
               CDR3
BrE-3 VH       ──────── WGQGTLVTVSA
Human III      ──────── .........S
```

Table 31 and 32 below contain the same information as Table 30 above in a different format. They show the numbers of the residues that would have to be changed in order to completely convert the original murine framework completely into a human consensus framework.

Table 31: BrE-3 V_L Amino Acid Candidates for
Change into Human Consensus Sequences

V_L			
VI2	FY36	EG68	GQ100
TS7	SP40	LV83	LV104
ST14	KQ45	FY87	LP15
DE17			
QP18			

Table 32: BrE-3 V$_H$ Amino Acid Candidates for
Change into Human Consensus Sequences

V$_H$		
KQ3	SA40	DN73
AS113	EV5	EG42
SN76	ML18	AS49
RT77	KR19	VL78
IN82a	GA88	LV89
		TA93
		GR94

Example 36: Identification of Important Murine BrE-3 Amino Acids

The "important" murine amino acids that should be preserved were chosen based on the contacts of a particular amino acid with the CDRs, and with the opposite chains and/or whether their side chains are pointing inwardly or outwardly. The positions of these "important" amino acids were determined based on the examination of the known structures of other antibodies. This information is provided in Tables 33 and 34 below.

Table 33: Important V$_L$ Amino Acid Positions to be Preserved

V$_L$		
V2	CDR contact	Buried
F36	CDR contact	Contact with VH
K45	CDR contact	
F87	Possible contact with VH	
G100	Possible contact with VH	
L104	Buried	

Table 34: Important V$_H$ Amino Acid Positions to be Preserved

V$_H$		
M18	Buried	
S40	Buried	
A49	CDR contact	Buried
D73	CDR contact	
S76		Buried
V78	CDR contact	Buried
L89	Might affect interaction with V$_L$	
T93	CDR contact	
G94	CDR contact	Buried

Most of the "important" amino acids were selected on the basis of the structure of antibody 4-4-20 and according to Tables 2, 3, 4, 5, 6, 8 and 9 above. Two important amino acids out of each chain, however, were selected based on more general structural analysis, using other antibody structures. This

5   was done to maximize the chances of conserving ligand binding properties. In particular the preservation of the Leu at 89-H was suggested in order to ensure the maintenance of, the $V_L:V_H$ contact. Although the residue aat 89-H is usually not in contact with V_L and is only partly buried, it nonetheless contributes to the interface. A Val for Leu replacement at this position could very well create a

10   "cavity" which could affect the contact. An Ile for Leu replacement would probably be fine since these amino acids have essentially the same side chain volume.

Finally, by comparing the position of all amino acids that are candidates for mutation, shown in Tables 31 and 32 above, with those that are "important"

15   and should be preserved, shown in Tables 33 and 34, the final selection of amino acid positions for actual mutation was attained. Any "important" amino acid position was eliminated from the list of candidates. Table 35 below shows the amino acids that were selected for change from murine to human identities to obtain the present humanized analogue.

20   Table 35: Selected Amino Acids for Mutation

V_L	V_H
TS7	KQ3
ST14	EV5
LP15	KR19
DE17	EG42
QP18	RT77
SP40	IN82a
EG68	GA88
LV83	AS113

Example 37:   Introduction of Changes in
              BrE-3 Amino Acid Sequence

35   The changes were done at the DNA level in sequential manner. All but one of the codon mutations were performed using enzymatic inverse PCR (EIPCR), a mutagenesis technique developed by Stemmer and Morris (Stemmer, W.P.C and Morris, S.K.,"Enzymatic Inverse Polymerase Chain Reaction: a Restriction Site Independent, Single Fragment Method for High Efficiency

40   Site-Directed Mutagenesis", BioTechniques 13:146-220 (1992)).

First, the entire plasmid, containing the target cDNA was amplified by inverse PCR using terminal mutagenic oligonucleotides. Second, BsaI was used to cut the ends of the incorporated primers. This enzyme cuts at a site that is displaced from its recognition site. Thus, after digestion of the

5 open amplified plasmid with BsaI, the BsaI recognition sequence was removed from the ends of the DNA. The DNA was left with complementary sticky ends and could be closed into a functional plasmid that contains the mutagenized region. The amino acid and DNA sequences of the non-mutated (wild-type) variable light and heavy chains of the anti-BrE-3 antibody are shown in Tables

10 15 and 16 above. The amino acid sequences of the anti-BrE-3 antibody frameworks and the mutations that were performed for the humanization, the oligonucleotide that was used for the mutagenesis, and the method of mutagenesis are shown in Tables 36 to 44 below.

Table 36: F$_{VL}$ FR1 Mutation Sites

Position	FR1	Analogue 1 (8 Changes)	DNA Codon (FR1) (Analogue)	Method for Mutagenesis	Primers
Leader Peptide Not Shown					
	D				
	V				
	V				
	M				
	T				
	Q				
7	T	S	ACT TCT	EIPCR*	(J037,J038)
	P				
	L				
	S				
	L				
	P				
	V				
14	S	T	AGT ACT	EIPCR	(J037,J038)
15	L	P	CTT CCT	EIPCR	(J037,J038)
	G				
17	D	E	GAT GAG	EIPCR	(J037,J038)
18	Q	P	CAA CCA	EIPCR	(J037,J038)
	A				
	S				
	I				
	S				
	C				

* EIPCR: Enzymatic Inverse Polymerase Chain Reaction

Table 37: F_{VL} FR2 Mutation Sites

Position	FR2	Analogue 1 (8 Changes)	DNA Codon (FR2) (Analogue)	Method for Mutagenesis	Primers
CDR1 Not Shown					
40	W F L Q K S G Q S P K L L I Y	P	TCA CCA	EIPCR*	(JO39,JO40)

* EIPCR: Enzymatic Inverse Polymerase Chain Reaction

Table 38: F_{VL} FR3 Mutation Sites

Position	FR3	Analogue 1 (8 Changes)	DNA Codon (FR3) (Analogue)	Method for Mutagenesis	Primers
CDR2 Not Shown					
68	G V P D R F S G S G S E T D F T L K I S R V E A E D	G	GAG GGG	EIPCR*	(JO41,JO42)
83	L G V Y F C	V	CTG GTG	EIPCR	(JO41,JO42)

* EIPCR: Enzymatic Inverse Polymerase Chain Reaction

Table 39: F_{VL} FR4 Mutation Sites

Position	FR4	Analogue 1 (10 Changes)	Analogue 2 (6 Changes)	DNA Codon (FR4) (Analogue)	Method for Mutagenesis	Primers
CDR3 Not Shown						
		F	F			
		G	G			
		G	G			
		G	G			
		T	T			
		K	K			
		L	L			
		E	E			
		I	I			
		K	K			

Table 40: F_{VH} FR1 Mutation Sites

Position	FR1	Analogue 1 (8 Changes)	DNA Codon (FR1) (Analogue)	Method for Mutagenesis	Primers
Leader Peptide Not Shown					
	E				
	V				
3	K	Q	AAG CAG	EIPCR*	(JO57,JO58)
	L				
5	E	V	GAG GTG	EIPCR	(JO57,JO58)
	E				
	S				
	G				
	G				
	G				
	L				
	V				
	Q				
	P				
	G				
	G				
	S				
	M				
19	K	R	AAA AGA	EIPCR	(JO57,JO58)
	L				
	S				
	C				
	A				
	A				
	S				
	G				
	F				
	T				
	F				
	S				

* EIPCR: Enzymatic Inverse Polymerase Chain Reaction

Table 41: F$_{VH}$ FR2 Mutation Sites

Position	FR2	Analogue 1 (8 Changes)	DNA Codon (FR2) (Analogue)	Method for Mutagenesis	Primers
CDR1 Not Shown					
	W				
	V				
	R Q				
	S				
	P				
42	E	G	GAG GGG	EIPCR*	(JO55,JO56)
	K				
	G				
	L				
	E				
	W				
	V				
	A				

* EIPCR: Enzymatic Inverse Polymerase Chain Reaction

Table 42: F$_{VH}$ FR3 Mutation Sites

Position	FR2	Analogue 1 (8 Changes)	DNA Codon (FR2) (Analogue)	Method for Mutagenesis	Primers
CDR2 Not Shown					
	R				
	F				
	T				
	I				
	S				
	R				
	D				
	D				
	S				
	K				
	S				
77	R	T	AGA ACT	EIPCR*	(JO53,JO54)
	V				
	Y				
	L				
	Q				
	M				
82	I	N	ATA AAT	EIPCR	(JO53,JO54)
	S				
	L				
	R				
	A				
	E				
	D				
	T				
88	G	A	GGC GCC	EIPCR	(JO53,JO54)
	L				
	Y				
	Y				
	C				
	T				
	G				

* EIPCR: Enzymatic Inverse Polymerase Chain Reaction

Table 43: F$_{VH}$ FR4 Mutation Sites

Position	FR2	Analogue 1 (8 Changes)	DNA Codon (FR2) (Analogue)	Method for Mutagenesis	Primers
CDR3 Not Shown					
	W				
	G				
	Q				
	G				
	T				
	L				
	V				
	T				
	V				
	S				
113	A	S	GCA TCT	PCR*	(JO51,JO52)*

**PCR: Polymerase Chain Reaction

Table 44: Sequences of Mutagenic Oligonucleotides

JO37 TCC CTG GGT CTC ACT CCT GGA GAG CCA GCT TCC ATC TCT TGC AGA TCT AGT (Seq. ID No. 50)

JO38 AGC TTG GGT CTC AGG AGT GAC AGG CAG GGA GAG TGG AGA TTG GGT CAT CAC AAC (Seq.ID No. 51)

JO39 G TTC CTG GTC TCG CCA GGC CAG TCT CCA AAG CTC CTG (Seq. ID No. 52)

JO40 T TGG AGG TCT CCC TGG CTT CTG CAG GAA CCA ATA TAA AT (Seq. ID No. 53)

JO41 TTC ACA GGT CTC ATC AGC AGA GTG GAG GCT GAG GAT GTG GGA GTT TAT TT (Seq. ID No. 54)

JO42 AGC CTC GGT CTC GCT GAT CTT GAG TGT GAA ATC TGT CCC TGA TCC ACT GC (Seq. ID No. 55)

JO51 CCT GGA GGA TCC ATG AGA CTC TCT TGT GCT (Seq. ID No. 56)

JO52 GTT GGG GCT AGC AGA AGA GAC AGT GAC CAG AGT (Seq. ID No. 57)

JO53 TAC CTG GGT CTC AAT AGC TTA AGA GCT GAA GAC ACT GCC CTT TAT TAC TGT (Seq. ID No. 58)

JO54 TTC AGC GGT CTC GCT ATT CAT TTG CAG GTA CAC AGT ACT TTT GGA ATC ATC (Seq. ID No. 59)

JO55 GTC CGC GGT CTC CCA GGG AAG GGG CTT GAG TGG GTT GCT GAA ATT AGA AA (Seq. ID No. 60)

JO56 CTC AAG GGT CTC CCC TGG AGA CTG GCG GAC CCA GTC CAT CCA GGC ATC A (Seq. ID No. 61)

JO57 T GAG GAG GTC TCA GGA GGC TTG GTG CAA CCT GGA GGA TCC ATG AGA CTC TCT (Seq. ID No. 62)

JO58 GG TTG CGG TCT CCC TCC TCC AGA CTC CAC AAG CTG CAC TTC ACT CTG GAC A (Seq. ID No. 63)

* note Primer set JO51 and JO52 was originally intended for mutagenizing K19 to R (by JO51) and A113 to S (by JO52), as described below. Primer JO51, however, was somehow defective (It did not run as a single band on a polyacrylamide gel) and thus only the A113 to S mutagenesis was successful. Mutation K19 to R was accomplished at a latter time with EIPCR, using primers JO57 and JO58.

Example 38: Synthesis of Primers

All primers except JO51 and JO52 were synthesized on a PCR-Mate EP DNA synthesizer model 391 (Applied Biosystems, Foster City CA). Primers JO51 and JO52 were purchased from Keystone Laboratories, Inc, Menlo Park, CA.

5 Both, the PCR method used with JO51 and JO52 and the EIPCR method used with all other primer sets, are described below.

The plasmid DNA template was extracted with a kit purchased from
QIAGEN (Tchapsworth, CA) and diluted to 1ng/μl in 10mM TRIS 1mM EDTA pH
7.5 - 8. This plasmid is composed of vector pCR1000 (Invitrogen Corporation,
San Diego, CA) into which the cDNA encoding the variable region to be

5   humanized was inserted.

A mixture of PCR primers was made where each primer was present at
a concentration of 10 pmole/μl in water.

The PCR amplification conditions were as follows. All reagents as well
as the GeneAmp PCR system 9600 were purchased from Perkin Elmer Cetus.

10   Optimal PCR conditions were determined empirically for each pair of mutagenic
primers. A matrix of conditions varying the concentration of $MgCl_2$, mutagenic
primers, and template plasmid DNA was set up. The annealing and extension
temperatures during PCR may be varied.

Plasmid template (500 pg/μl), 0.5 μM each mutagenic oligonucleotide,

15   1 mM $MgCl_2$, 10 mM TRIS pH 8.3, 50 mM KCl,   0.2 mM each nucleotide
triphosphate (dGTP, dATP, TTP, dCTP), and Taq polymerase 1 unit/ 20μl
reaction mixture.

Example 39: Hot Start PCR

All the components of the PCR mixture, with the exception of Taq

20   polymerase, were mixed in a 95 μl volume. The mixture was then dispensed in
19 μl aliquots into 5 PCR tubes. The reason for performing five independent
reactions was to decrease the odds that unwanted mutations be isolated as a
result of nucleotide misincorporation during PCR. The tubes were heated to
95°C for 5 minutes and then cooled to 72°C. While at that temperature 1 μl

25   of an appropriate Taq polymerase dilution in 10 mM TRIS, pH 8.3, 50 mM KCl
was added to the reaction tubes. The temperature cycling then proceeded as
follows.
94°C, 3 min
[(94°C, 1 min) (50°C, 1 min) (72°C, 3.5 min)] 3 cycles

30   [(94°C, 1 min) (55°C, 1 min) (72°C, 3.5 min)] 25 cycles
72°C, 10 min

Example 40: Extra Final Extension

After cycling, the contents of the five tubes were mixed and an extra
final extension reaction was carried out. Extra nucleotide triphosphates were

35   added (to 125 μM). 5 units of Taq polymerase were also added and the mixture
was heated to 72°C for 10 minutes.

Example 41: Purification of PCR Products

The PCR products were then separated on a 0.8% agarose gel in 1XTAE buffer and 0.5 µg/ml Ethidium Bromide. The correct DNA band was visualized with UV light (366 nm), excised from the gel and extracted with the GeneClean
5 kit purchased from Bio 101, La Jolla, CA.

Example 42: Restriction Digestion

The DNA was then digested with BsaI for two hours at 60 degrees celsius in 25 µl (20.5 µl of DNA, 2.5 µl 10 X buffer 4 (NEB) and 2 µl BsAI (NEB). BsaI sites were designed near the 5' end of the PCR primers. The primers
10 included 6 extra nucleotides 5' of the BsaI sites to facilitate digestion by BsaI. There were no BsaI sites elsewhere in the plasmid. Other restriction enzymes may be used as advised by Stemmer and Morris (Stemmer and Morris (1992), supra). This special class of restriction enzyme cuts at a site that is different from its recognition site but, nevertheless, at a precise distance from it. Using
15 this method, there was no need for having restriction sites in the sequence in order to perform the mutagenesis.

Example 43: Second Purification

The restricted products were then separated on a 0.8% agarose gel in 1XTAE buffer and 0.5 µg/ml Ethidium Bromide. The correct DNA band was
20 visualized with UV light (366 nm), excised from the gel and extracted with the GeneClean kit purchased from Bio 101, La Jolla, CA.

Example 44: Ligation (Reclosure of Plasmid)

The ligation mixtures consisted of 5 µl extracted DNA, 2 µl 10x ligation buffer (NEB) 1 µl T4 DNA polymerase (NEB), 12 µl water. The amount of
25 plasmid DNA may be varied depending of the intensity of the band extracted from the Gel. Ligation was carried out at room temperature for 2 hrs., or alternatively at 14°C overnight.

Example 45: Transformation and Sequencing

The reclosed plasmids were then transformed into E. coli. We used Inv
30 alpha F' competent cells purchased from Invitrogen Corporation, San Diego CA.

Plasmid DNA was then prepared from a few transformants and sequenced to verify that mutagenesis was successful.

Example 46: Mutagenesis of BrE-3 Antibody using JO51 and JO52

These oligonucleotides were designed to mutagenize K19 to R (by JO51)

79

and A113 to S (by JO52), not by using EIPCR but using the normal PCR (with primers pointing to each other). Primer JO51 carried a BamHI site and primer JO52 carried an NheI site. After mutagenic PCR amplification the resulting amplified DNA cassette was inserted into the plasmid in lieu of the corresponding

5   wild-type DNA fragment. There was no compelling reason to use this method over the EIPCR method except that conveniently placed restriction sites (BamHI and NheI) were available. This method, however, yielded only the A113 to S mutation. A subsequent analysis showed that the JO51 primer, which carried the K19 to R mutation ran aberrantly on a polyacrylamide gel.

10   The protocol for the mutagenic amplification step was as follows. Plasmid template (500 pg/μl), 0.75 μM each mutagenic oligonucleotide, 2 mM MgCl$_2$, 10 mM TRIS pH 8.3, 50 mM KCl, 0.2 mM each nucleotide triphosphate (dGTP, dATP, TTP, dCTP), and Taq polymerase 1 unit/20 μl reaction mixture..

The PCR was hot started as described for EIPCR above. The temperature

15   cycling conditions used were as follows.

94°C, 3 min

[(94°C, 1 min) (44°C, 1 min) (72°C, 1 min)] 3 cycles

[(94°C, 5 sec) (65°C, 1 min) (72°C, 1 min)] 32 cycles

72°C, 10 min

20   The extra final extension and purification of PCR products were conducted as described for EIPCR above.

The restriction digestion of the vector and the insert were conducted as follows. The PCR product was digested with BamHI and NheI for 1 hr. 50 min. at 37°C (19 μl of DNA, 2.5 μl 10Xbuffer 3 (NEB), 1.5 μl BamHI (NEB), 1.5 μl

25   NheI (NEB). The vector, which is the starting plasmid described above, was digested under similar conditions [1μl plasmid (1 μg), 2 μl 10Xbuffer 3 (NEB), 1.5 μl BamHI (NEB), 1.5 μl NheI (NEB) 14 μl water, 2½hrs. at 37°C].

The restricted products, vector and insert, were then purified once more as described above for EIPCR.

30   The ligation of the fragments was conducted as follows. The ligation mixtures consisted of 5 μl vector, 5 μl insert, 2 μl 10x ligation buffer (NEB) 1 μl T4 DNA polymerase (NEB), 7 μl water. The amount of plasmid DNA may be varied depending of the intensity of the band extracted from the Gel. Ligation is carried out at 14°C overnight. A control ligation with vector only was carried

35   out in parallel. The transformation of the host cells was conducted as described for EIPCR.

Example 47: Plasmid Preparation and Sequencing of Humanized BrE-3 V$_H$

Plasmid DNA was then prepared from several independent transformants. A few of the plasmids that were shown by restriction analysis to contain the insert were then sequenced to verify that the mutagenesis was successful. When one of the sequenced DNAs contained the desired mutation

5. it was utilized for the next mutation cycle. The fully mutated humanized analogue BrE-3 DNA sequences for the V$_H$ and V$_L$ segments are shown in Tables 45 and 46 below.

Table 45: BrE-3 Antibody V$_L$ Humanized Analogues DNA Sequences
BrE-3 V$_L$ FR-HZ

10

```
ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG TTG TTC TGG ATT CCT GCT TCC ATC AGT GAT GTT GTG
ATG ACC CAA TCT CCA CTC TCC CTG CCT GTC ACT CCT GGA GAG CCA GCT TCC ATC TCT TGC AGA TCT
AGT CAG AAC CTT GTA CAC AAC AAT GGA AAC ACC TAT TTA TAT TGG TTC CTG CAG AAG CCA GGC CAG
TCT CCA AAG CTC CTG ATT TAT AGG GCT TCC ATC CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC
```

15 ```
AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT GTG GGA GTT TAT
TTC TGC TTT CAA GGT ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA C
```
(Seq. ID No. 64)

**Table 46:** BrE-3 Antibody VH Humanized Analogue DNA Sequences

**BrE-3 VH FR-HZ**

20 ```
ATG TAC TTG GGA CTG AAC TAT GTC TTC ATA GTT TTT CTC TTA AAA GGT GTC CAG AGT GAA GTG CAG
CTT GTG GAG TCT GGA GGA GGC TTG GTG CAA CCT GGA GGA TCC ATG AGA CTC TCT TGT GCT GCT TCT
GGA TTC ACT TTT AGT GAT GCC TGG ATG GAC TGG GTC CGC CAG TCT CCA GGG AAG GGG CTT GAG TGG
GTT GCT GAA ATT AGA AAC AAA GCC AAT AAT CAT GCA ACA TAT TAT GAT GAG TCT GTG AAA GGG AGG
TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT ACT GTG TAC CTG CAA ATG AAT AGC TTA AGA GCT GAA
```
25 ```
GAC ACT GCC CTT TAT TAC TGT ACT GGG GAG TTT GCT AAC TGG GGC CAG GGG ACT CTG GTC ACT GTC
TCT TCT G (Seq. ID No.: 65)
```

**Example 48:** Humanized BrE-3 Antibody Expression

Two expression vectors pAG4622 and pAH4604 (Coloma, M.J., et al. (1992), supra) were used that were developed and provided by S.L. Morrison

30 (Dept. of Microbiology and Molecular Genetics, UCLA). Any cDNA encoding a signal peptide and either the variable heavy chain or the variable light chain can, in principle, be inserted into these vectors resulting in a construction that encodes an IgG1, K, antibody with human constant regions. Correctly modified cDNAs were excised from pCR1000 with EcoRV and Sal I and inserted into

35 pAG4622. These encode the modified light chain. The wild-type heavy chain

was similarly excised from pCR1000 by digestion with EcoRV and NhEI and inserted into pAH4604. The restriction and ligation reactions necessary to accomplish these operations were performed under the conditions stipulated by the enzyme manufacturers (New England Biolabs, Beverly, MA). Both the

5    vectors and the inserts were purified from an agarose gel prior to ligation, using the Bio101 (La Jolla, CA) GeneClean kit (glass beads). The $V_H$ and $V_L$ regions in the final constructions were sequenced once again to verify that they are correct. The non-producer myeloma cell line SP2/0-Ag14, ATCC: CRL 1581, (Shulman M., et al. (1978), supra) was transfected with both plasmid

10   constructions, and antibody producers were isolated following the recommendations outlined in (Coloma, M.J. et al. (1992), supra) except that selection was done only for the uptake of hisD (by adding 5 mM histidinol to the medium and readjusting the pH to 7.4 with NaOH). Usually after ten days, stable transfectant colonies were established at a frequency of approximately

15   $10^{-5}$ to $10^{-4}$. Colonies were then transferred to normal medium (without histidinol). The culture media were either Dulbeco's modified Eagle's medium (DME): fetal bovine serum (FBS), 90:10, v/v, or a mixture of DME:RPMI:FBS, 45:45:10, v/v/v. Penicillin and streptomycin were added to prevent bacterial growth.

20       The supernatants from stable transfectants were assayed for the presence of the antibodies. This was done by capturing the secreted chimeric antibody with a plate-bound goat anti-human-kappa chain antibody and developing with goat anti-human-gamma chain antibody, essentially as described previously (Coloma, M.J. (1992), supra) except that the secondary antibody was

25   radiolabeled with [125]I. The supernatants were also assayed for binding to human milk fat globule (HMFG) as described previously (Ceriani R.L., et al., "Diagnostic Ability of Different Human Milk Fat Globule Antigens in Breast Cancer", Breast Cancer Res. Treat., 15:161-174 (1990)). HMFG is bound to the microtiter plates as described previously (Ceriani, R.I. (1984), supra). Usually most colony

30   supernatants were positive by both assays. Colonies that secrete the highest level of antibody in the supernatants, as determined by these assays, were subcloned and subsequently adapted to serum-free medium for the purification of antibody. Serum free medium contains HL-1 supplement as directed by the manufacturer (Ventrex Labs., Portland, ME).

35   Example 49: Half Humanized-Half Chimeric BrE-3 Antibody

         A BrE-3 humanized light chain was paired with an anti-BrE-3 non-humanized chimeric heavy chain by co-transfection of SP2/0 myeloma cells with hybrid plasmids carrying the respective DNA sequences and those of a human

82

$F_c$.

<u>Example 50</u>:     Determination of Affinity Constants for Half
                              Humanized and Fully Humanized BrE-3 Antibodies
                      The secreted half humanized-half chimeric and fully humanized antibodies

5    were purified from culture supernatants using a Sepharose 4B-protein A column
(Bio-Rad, Richmond, CA.) as described by Ey et al. (Ey, P.L., et al. (1978),
supra). Microtiter plates (Dynatech, Chantilly, VA) were prepared as described
by Ceriani et al. (Ceriani, R.L., et al. (1992), supra) using successive layers of
methylated BSA, glutaraldehyde, anti-$\beta$-galactosidase and the bacterial fusion

10   protein 11-2 (a hybrid of $\beta$-galactosidase and human mammary mucin). Each
well contained 388 ng of the 11-2 fusion protein. To each well were added 25
$\mu$l $^{125}$I -BrE-3 in RIA buffer (10 % bovine calf serum, 0.3% triton X-100, 0.05
% sodium azide pH7.4, in phosphate buffer saline) and compete with 25 $\mu$l of
either unlabeled murine or chimeric antibody in RIA buffer at the final

15   concentrations of 130 pM, 850 pM, 1.3 nM, 4 nM, and 13 nM). Iodinations
were performed with $^{125}$I (17Ci/mg, Nordion International). Fifty micrograms of
monoclonal antibody BrE-3 (Coulter, Hialeah, FL) were labeled at a specific
activity of 9.56 mCi/mg using the chloramine T method as described previously
by Ceriani et al. (Ceriani, R.L., et al. (1988), supra)

20       Antibody-antigen affinity constants were determined by taking the
reciprocal of the concentration of competing unlabeled monoclonal antibody that
produced 50 % binding as described by Sheldon et al. (Sheldon, K., et al.
(1987), supra). The protocol used to determine affinity constants was as
described above except that in each case, an unlabeled antibody competed for

25   binding to antigen against the same radiolabeled antibody. Both, the half
humanized-half chimeric antibody and the fully humanized antibody competed
about as well or better with the anti-BrE-3 murine antibody for the antigen.

         Polyacrylamide gel electrophoresis was performed to insure that the
antibody chains migrated as expected. The affinity binding constants of the

30   murine, chimeric, half humanized and humanized antibodies were determined in
independent competition assays.

<u>Example 51</u>:     Histochemical Specificity of Half and
                              Fully Humanized BrE-3 Antibodies
                      Immunohistochemical staining using the immunoperoxidase technique

35   of consecutive human breast carcinoma tissue sections was used as a test to
verify that the analogue antibodies retain useful affinity for the carcinoma
antigens. Breast carcinoma tissue sections were stained with the supernatant
of the half humanized/half chimeric and fully humanized transfected cells using

the Vectastain ABC method (Vector Labs, Burlingame, CA). Both antibodies showed strong staining patterns.

Example 52:    Gel Chromatography of Half Humanized-Half
                Chimeric and Fully Humanized BrE-3 Antibodies

5       Antibody disulfide bonds were reduced by heating for 10 min at 65°C in Laemmli loading buffer containing 5% beta-mercaptoethanol. The separated chains were then chromatographed on a SDS polyacrylamide gel (10%). Two bands were observed for both antibodies of similar migration pattern as the murine antibody. These data were also confirmed by Western blotting.

10      Example 53:    Deduced Amino Acid Sequences of Humanized
                        BrE-3 Variable Light and Heavy Chains

        The amino acid sequences of the light and heavy chains of the analogue humanized antibody are shown in Tables 47 and 48 below. These amino acid sequences may be improved either to increase affinity for the antigen or to

15      decrease immunogenicity in humans. Numerous variants of this sequence may be engineered in accordance with the invention.

Table 47: Humanized BrE-3 V_L Analogue Amino Acid Sequence
BrE-3 V_L FR-HZ

| | |
|---|---|
| 20   Leader | mklpvrllvlLFWIPASIS (Seq. ID No: 66) |
| FR1 | DVVMTQSPLSLPVTPGEPASISC (Seq. ID No: 67) |
| CDR1 | RSSQNLVHNNGNTYLY (Seq. ID No: 68) |
| FR2 | WFLQKPGQSPKLLIY (Seq. ID No: 69) |
| CDR2 | RASIRFS (Seq. ID No: 70) |
| 25   FR3 | GVPDRFSGSGSGTDFTLKISRVEAEDVGVYFC (Seq. ID No: 71) |
| CDR3 | FQGTHVPWT (Seq. ID No: 72) |
| FR4 | FGGGTKLEIK (Seq. ID No: 73) |

Table 48: BrE-3 V_H Analogue Amino Acid Sequence
BrE-3V_H FR-HZ

| | |
|---|---|
| Leader | mylglnyvflVFLLKGVQS (Seq. ID No: 74) |
| FR1 | EVQLVESGGGLVQPGGSMRLSCAASGFTFS (Seq.ID No: 75) |
| 35   CDR1 | DAWMD (Seq. ID No: 76) |
| FR2 | WVRQSPGKGLEWVA (Seq. ID No: 77) |
| CDR2 | EIRNKANNHATYYDESVKG (Seq. ID No: 78) |
| FR3 | RFTISRDDSKSTVYLQMNSLRAEDTALYYCTG (Seq. ID No: 79) |
| CDR3 | EFAN (Seq. ID No: 80) |
| 40   FR4 | WGQGTLVTVSS (Seq. ID No: 81) |

Example 54: Choice of Murine Model of Known Structure
           for Humanization of anti-KC-4 Antibody

The classification of the $V_H$ and $V_L$ domains of an antibody such as the
anti-KC-4 antibody was done according to Kabat et al. (Kabat, E.A., et al.,
5   "Sequences of Proteins of Immunological Interest" NIH (1991). The KC-4G3
kappa chain $V_L$ domain belongs to group II and the $V_H$ domain belongs to group
IIId. A murine antibody was then found, whose structure had been determined,
and whose variable regions belong to the same classes.   The anti-
myohemerythrin peptide antibody B13I2 fits these requirements since, like the
10  anti-KC-4 murine antibody, it has $V_L$ and $V_H$ domains belonging to groups II and
IIId (Stanfield, R.L., et al., "Crystal Structures of an Antibody to a Peptide and
its complex with Peptide Antigen at 2.8 Å", Science 248:712-719 (1990)).
Thus, the three-dimensional structures of antibodies the anti-KC-4 and B13I2
antibodies should be similar, and the humanization of the anti-KC-4 antibody may
15  be modeled after B13I2.


Example 55:     Choice of Target Human Framework for
                Humanization of Chimeric anti-KC-4 Antibody

The choice of the target human framework was based strictly on the
similarity at the residues that were judged to be structurally important according
20  to the B13I2 model. That is, only amino acids that could be involved in contacts
with CDRs of the opposite chain, or amino acids whose side-chains were
predicted to be inwardly pointed. The positions of these amino acids are shown
in Tables 49 below.

Table 49: Important Amino Acid Positions for anti-KC-4 Antibody
25  _____

Light Chain Variable Region Framework

2, 3, 4, 6, 7, 11, 13, 19, 21, 22, 23, 35, 36, 37, 38, 43,
44, 46, 47, 48, 49, 58, 60, 61, 62, 69, 71, 73, 75, 78,
82, 85, 86, 87, 88, 98, 102, 104 and 106.

30  Heavy Chain Variable Region Framework

2, 4, 6, 12, 18, 20, 22, 24, 27, 28, 29, 36, 37, 38, 39, 43, 45, 46, 47, 48,
49, 66, 67, 69, 71, 78, 80, 82, 82c, 86, 88, 90, 91, 92, 93, 94, 103, 107,
109 and 111.

_____

35  The numbering system is conventionally accepted (Kabat, et al. (1991),
supra) and is shown in Tables 10 and 11 above. In this case, the consensus
sequences of all human $F_v$ regions were selected as the target human framework
to minimize the immunogenicity of the product.

First, the sequences of the murine variable chains were aligned with consensus sequences from all known human variable region classes (Herron, J.N., (1989), supra) and the number of differences in the amino-acids that must be retained from the murine were scored. The positions of these amino acids

5    were obtained from those of the B1312 murine monoclonal antibody, which was chosen to model the humanization of the anti-KC-4 antibody.

Based on these scores, the consensus sequences human frameworks belonging to groups $V_k II$ and $V_H III$ were chosen to receive the anti-KC-4 murine antibody CDRs plus other important amino acids.

10   **Example 56:**        Identification of Murine-Human
                         anti-KC-4 Antibody Differences
        The original murine sequences (anti-KC-4 $V_k$ or $V_H$) were aligned with their closest human (Human KII or HIII) relatives that were chosen after comparing their sequences as described in Example 34 above. In the present

15   example, it was intended to be substituted as many amino acids as possible in going from the murine to the humanized variable consensus sequences, leaving the important amino acids intact as described in Examples 55 and 57. The amino acids chosen to be preserved were a subset of those listed above. These were selected by analogy to the B1312 sequence. The single exception was the

20   glycine (100) residue of the original framework of the variable region of the murine kappa chain, which was retained despite not being encompassed in Table 49 above since it was thought that it might contact the variable domain of the heavy chain. Such contacts were observed in at least three Fab that lack a gly at this position.

25   **Example 57:**        Identification of Important Murine
                         anti-K-4 Antibody Amino Acids
        The "important" murine amino acids were chosen for preservation based on the contacts of a particular amino acid with the CDRs, and with the opposite chains and/or whether their side chains are pointing inwardly or outwardly. The

30   positions of these "important" amino acids were determined based on the examination of the known structures of other antibodies.

        Most of the "important" amino acids were selected on the basis of the structure of antibody B1312 and according to Tables 2, 3, 4, 5, 6, 8 and 9 above. The final selection of amino acid positions for actual mutation was

35   attained by comparing the position of all amino acids that are candidates for mutation with those that are "important" and should be preserved. Any "important" amino acid position was eliminated from the list of candidates. Table 50 below shows the amino acids that were selected for change in the

murine sequence to attain the humanized sequence in the present exemplary analogue.

**Table 50: Anti-KC-4 Murine Antibody Variable Region
Amino Acids Selected for Mutation**

| Position | KC-4G3 Murine Identity ---> | Consensus Human Identity |
|---|---|---|
| **Light Chain Variable Region** | | |
| 14 | S | T |
| 15 | L | P |
| 17 | D | E |
| 18 | Q | Q |
| 45 | K | K |
| 74 | N | D |
| 83 | L | V |
| **Heavy Chain Variable Region** | | |
| 13 | K | Q |
| 19 | K | R |
| 40 | S | A |
| 42 | R | G |
| 74 | A | G |
| 81 | E | S |
| 82a | S | N |
| 84 | N | A |
| 89 | M | V |
| 110 | S | Y |
| 113 | A | S |

The change N-----> K at position 74 in the variable light chain knowingly eliminated an N-linked glycosylation site, which was present in the original murine monoclonal antibody.

**Example 58:    Introduction of Changes in Amino Acid Sequence
for Humanization of anti-KC-4 Antibody**

The introduction of the changes in the amino acid sequence was not done as described in Example 37 above.   Instead the DNA encoding each humanized variable region was synthesized in a single polymerase chain reaction (PCR) using overlapping oligonucleotides in accordance · with the method described by Ye et al. (Ye, Q-Z, Jonhson, L.L., and Baragi, V., "Gene Synthesis and Expression in E. coli for PUMP, a Human Matrix Metalloproteinase", BBRC 186(1):143-149 (1992)). The sequences of the mutagenic are shown in Table 51 below.

Table 51: Primers for Humanization of anti-KC-4
Murine Antibody Variable Regions

| | |
|---|---|
| JA59 | CCCGGATCC TTTAAAAGGT GTCCAGTGTG AAGTGCAGAT GGTGGAG TCTG (SEQ.ID No:82) |
| J060 | GAATTCGGGGC TAGCACTAGA GACAGTGACC AGAGTCCCTT GGCCCCAG (SEQ.ID No:83) |
| J061 | AGTGCAGATG GTGGAGTCTG GGGGAGGCTT AGTGCAGCCT GGAGGGTCCC TGAGACTCTC CTGTGCAGCC TCTGGATTCG CTTTCAGTAG CTATGCCATG T(SEQ.ID No:84) |
| J062 | CTTGATAGTA GGCGTAATTA CCACCACTAC TAATTTCTGC GACCCACTCC AGCCCCTTCC CTGGAGCCTG GCGAACCCAA GACATGGCAT AGCTACTGAA A (SEQ. ID. No.: 85) |
| J063 | TAATTACGCC TACTATCAAG ACACTGTGAC GGGCCGATTC ACCATCTCCA GAGACAATTC CAAGAACACC CTGTACCTGC AAATGAACAG TCTGAGGGCT G (SEQ. ID. No.: 86) |
| J064 | CCAGAGTCCC TTGGCCCCAG TAAGCAAACC AGGCCGGGAT ACCGTAGTCC TCCCTTGCAC AGTAATACAC GGCCGTGTCC TCAGCCCTCA GACTGTTCAT T (SEQ. ID. No.: 87) |
| J073 | GGGAAGCTTG ATATCCACCA TGAAGTTGCC TGTTAGGCTG TTGGTGCTGA TGTTCTGGAT TCCTGC (SEQ. ID No.: 88) |
| J074 | AAAGATTCG TCGACTTACG TTTTATTTCC AGCTTGGTCC CCCCTCC GAA CGTGTACGGA ACATGT (SEQ. ID. No.: 89) |
| J075 | CTGATGTTCT GGATTCCTGC TTCCAGCAGT GATGTTTTGA TGACCC AAAC TCCTCTCTCC CTGCCTGTCA CTCCAGGAGA GCCAGCCTCC ATCTCTTGCA (SEQ. ID. No.: 90) |
| J076 | CTGTGGAGAC TGGCCTGGTT TCTGCAGGTA CCATTCTAAA TAGGTG TTTC CATTACTATG TACAATGCTC TGACTAGATC TGCAAGAGAT GGAGGCTGGC (SEQ. ID. No.: 91) |
| J078 | CGAACGTGTA CGGAACATGT GAACCTTGAA AGCAGTAATA AATTCC CACA TCCTCAGCCT CCACTCTGCT GATCTTGAGT GTGAAATCTG TCCCTGATCC (SEQ.ID. No.: 92) |

Example 59:     Synthesis of Primers for Humanization
of anti-KC-4 Antibody

All primers were synthesized on a PCR-Mate EP DNA synthesizer model
391 (Applied Biosystems, Foster City CA) using 40nmole columns, cycle 1:63,
with Trityl off.  None were purified before use.  The EIPCR method was used
for preparing all primer sets.  Their sequences are shown in Table 51 above.

The plasmid DNA template was extracted with a kit purchased from
QIAGEN (Tchapsworth, CA) and diluted to 1ng/μl in 10mM TRIS 1mM EDTA pH
7.5 - 8.  This plasmid is composed of vector pCR1000 (Invitrogen Corporation,
San Diego, CA) into which the cDNA encoding the variable region to be
humanized was inserted.

Example 60:     Synthesis of anti-KC-4 Humanized
Heavy Chain Variable Regions

A mixture of PCR primers was made, where each primer was present at
a concentration of 10 pmole/μl in water.

Four 101 'mer oligonucleotides (JO61, JO62, JO63 and JO64), one
50'mer (JO59), and one 49'mer (JO60), were used for the synthesis of the
humanized variable heavy chain.  The oligonucletides  concentrations were

estimated using the formula

$$c = [(A_{260})/30]\mu g/\mu l$$

The PCR amplification conditions were as follows. All reagents as well as the GeneAmp PCR system 9600 were purchased from Perkin Elmer Cetus.

5     Optimal PCR conditions were determined empirically for each pair of mutagenic primers. A matrix of conditions varying the concentration of $MgCl_2$, mutagenic primers, and template plasmid DNA were set up as follows. However, the annealing and extension temperatures during PCR may be varied.

$2\mu M$ primer JO59    150nM each of primers JO61, 62, 63 and 64.
10    $2\mu M$ primer JO60    200$\mu M$ each of dGTP, dATP, TTP, and dCTP.
10mM KCl             20mM Tris-HCl pH 8.8
10mM $(NH_4)_2SO_4$    2 units per 100$\mu l$ reaction Vent DNA
0.1% Triton X-100   polymerase (New England Biolabs)
                     6mM $MgSO_4$

15    <u>Example 61</u>: Hot Start PCR for Humanization of anti-KC-4 Antibody

All the components of the PCR mixture, with the exception of Vent DNA polymerase, were mixed. The mixture was then dispensed in 19 $\mu l$ aliquots into 5 PCR tubes. The reason for performing five independent reactions was to decrease the odds that unwanted mutations be isolated as a result of nucleotide

20    misincorporation during PCR. The tubes were heated to 95°C for 5 minutes and then cooled to 72°C. While at that temperature 1 $\mu l$ of an appropriate Vent DNA polymerase dilution in 1 x buffer was added to the reaction mixture (hot start). The temperature cycling then proceeds as follows.

[(96°C, 6 sec) (55°C, 10 sec) (72°C, 30 sec)] 3 cycles
25    [(96°C, 5 sec) (60°C, 10 sec) (72°C, 30 sec)] 29 cycles
72°C, 10 min

<u>Example 62</u>:      Extra Final Extension for Humanized
                    anti-KC-4 Antibody DNA
     After cycling, one extra final extension reaction was carried out. Extra
30    deoxyribonucleotide triphosphates (to 125 $\mu M$) and 1 unit of Vent DNA polymerase were added, and the mixture was heated to 72°C for 10 minutes.

The resulting synthetic DNA fragment was digested with DraI and NheI and inserted into the same restriction sites a plasmid construct encoding the corresponding murine heavy chain variable region.

35    <u>Example 63</u>:      Synthesis of anti-KC-4 Humanized
                    Light Chain Variable Regions

The light chain variable region ($V_L$) genes were synthesized in a similar way as described in Examples 60 to 62 above for the heavy chain variable regions.

<u>Example 64</u>:    Purification of Humanized anti-KC-4 PCR Products

The PCR products were then separated on a 0.8% agarose gel in 1XTAE buffer and 0.5 μg/ml ethidium bromide. The correct DNA band were visualized with UV light (366 nm), excised from the gels and extracted with the GeneClean

5    kit (Bio 101, La Jolla, CA).


<u>Example 65</u>:       Litigation of Humanized anti-
                        KC-4 DNA to Plasmid
                        (Reclosure of Plasmid)

The ligation mixtures consisted of 5 μl extracted DNA, 2 μl 10x ligation

10   buffer (NEB) 1 μl T4 DNA polymerase (NEB), 12 μl water. The amount of plasmid DNA may be varied depending of the intensity of the band extracted from the Gel. Ligation into a pBluescript KS⁺ plasmid (Stratagene) was carried out at room temperature for 2 hrs., or alternatively at 14°C overnight.


15   <u>Example 66</u>:       Transformation and Sequencing
                        of Humanized anti-KC-4 DNA

The reclosed plasmids were then transformed into E. coli utilizing Inv alpha F' competent cells purchased from Invitrogen Corporation, San Diego CA. Plasmid DNA was then prepared from a few transformants and sequenced to

20   verify that mutagenesis was successful.


<u>Example 67</u>:       Hybrid Plasmid Preparation and Sequencing

Plasmid DNA was then prepared and sequenced to verify that the mutagenesis was successful. The mutated anti-KC-4 humanized analogue DNA sequences for the $V_H$ and $V_L$ segments are shown in Tables 52 and 53 below.


25   <u>Table 52</u>:       Humanized anti-KC-4 Antibody $V_L$
                        Analogue DNA Sequences

```
                            anti-KC-4 V_L FR-HZ
     ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG ATG TTC TGG ATT CCT GCT
     TCC AGC AGT GAT GTT TTG ATG ACC CAA ACT CCT CTC TCC CTG CCT GTC
30   ACT CCA GGA GAG CCA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT
     GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
     GGC CAG TCT CCA CAG CTC CTG ATC TAC AAA GTT TCC ATC CGA TTT TCT
     GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA
     CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT GTG GGA ATT TAT TAC TGC
35   TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTG
     GAA ATA AAA C (SEQ. ID. No.: 93)
```

Table 53:   Humanized anti-KC-4 Antibody V_H
Analogue DNA Sequences

anti-KC-4 V_H FR-HZ
ATG GAC TTT GGG CTC AGC TTG GTT TTC CTT GTC CTT ATT TTA AAA GGT
5 GTC CAG TGT GAA GTG CAG ATG GTG GAG TCT GGG GGA GGC TTA GTG CAG
CCT GGA GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC GCT TTC
AGT AGC TAT GCC ATG TCT TGG GTT CGC CAG GCT CCA GGG AAG GGG CTG
GAG TGG GTC GCA GAA ATT AGT AGT GGT GGT AAT TAC GCC TAC TAT CAA
GAC ACT GTG ACG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC
10 ACC CTG TAC CTG CAA ATG AAC AGT CTG AGG GCT GAG GAC ACG GCC GTG
TAT TAC TGT GCA AGG GAG GAC TAC GGT ATC CCG GCC TGG TTT GCT TAC
TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT AGT (SEQ. ID. No.: 94)

Example 68: Expression of Anti-KC-4 Humanized Antibody

Two expression vectors pAG4622 and pAH4604 (Coloma, M.J., et al.
15 (1992), supra) were used that were developed and provided by S.L. Morrison
(Dept. of Microbiology and Molecular Genetics, UCLA). Any cDNA encoding a
signal peptide and either the variable heavy chain or the variable light chain can,
in principle, be inserted into these vectors resulting in a construction that
encodes an IgG1, K, antibody with human constant regions. Correctly modified
20 cDNAs were excised from pCR1000 with EcoRV and Sal I and inserted into
pAG4622. These encode the modified light chain. The wild-type heavy chain
was similarly excised from pCR1000 by digestion with EcoRV and NhEI and
inserted into pAH4604. The restriction and ligation reactions necessary to
accomplish these operations were performed under the conditions stipulated by
25 the enzyme manufacturers (New England Biolabs, Beverly, MA).   Both the
vectors and the inserts were purified from an agarose gel prior to ligation, using
the Bio101 (La Jolla, CA) GeneClean kit (glass beads). The V_H and V_L regions
in the final constructions were sequenced once again to verify that they were
correct. The non-producer myeloma cell line SP2/0-Ag14, ATCC: CRL.1581,
30 (Shulman M., et al. (1978), supra) was transfected with both plasmid
constructions, and antibody producers were isolated following the
recommendations outlined in Coloma et al. (Coloma, M.J. et al. (1992), supra)
except that selection was done only for the uptake of hisD (by adding 5 mM
histidinol to the medium and readjusting the pH to 7.4 with NaOH). Usually after
35 ten days, stable transfectant colonies were established at a frequency of
approximately 10^{-5} to 10^{-4}. Colonies were then transferred to normal medium
(without histidinol). The culture media were either Dulbeco's modified Eagle's
medium (DME): fetal bovine serum (FBS), 90:10, v/v, or a mixture of
DME:RPMI:FBS, 45:45:10, v/v/v. Penicillin and streptomycin were added to
40 prevent bacterial growth.

The supernatants from stable transfectants were assayed for the
presence of the antibodies. This was done by capturing the secreted chimeric

antibody with a plate-bound goat anti-human-kappa chain antibody and developing with goat anti-human-gamma chain antibody, essentially as described previously (Coloma, M.J. (1992), supra) except that the secondary antibody was radiolabeled with [125]I. The supernatants were also assayed for binding to human

5 milk fat globule (HMFG) as described previously (Ceriani R.L., et al., "Diagnostic Ability of Different Human Milk Fat Globule Antigens in Breast Cancer", Breast Cancer Res. Treat., 15:161-174 (1990)). HMFG is bound to the microtiter plates as described previously (Ceriani, R.I. (1984), supra). Usually most colony supernatants were positive by both assays.   Colonies that secrete the

10 highest level of antibody in the supernatants, as determined by these assays, were subcloned and subsequently adapted to serum-free medium for the purification of antibody. Serum free medium contains HL-1 supplement as directed by the manufacturer (Ventrex Labs., Portland, ME).

Example 69: Half Humanized-Half Chimeric anti-KC-4 Antibody

15 An anti-KC-4 humanized light chain was paired with an anti-KC-4 non-humanized chimeric heavy chain by co-transfection of SP2/0-Ag14 myeloma cells with hybrid plasmids carrying the respective DNA sequences and those of a human F_c. The cell line obtained, HuKC-4V1 was deposited with the ATCC on September 23, 1993 and awarded an accession number HB 11454.

20 In addition, an anti-KC-4 humanized heavy chain was paired with an anti-KC-4 non-humanized chimeric light chain as described in Example 69 above. The thus obtained cell, HuKC-4V3 was deposited with the ATCC on September 23, 1993 and awarded an accession number HB 11456.

Example 70: Fully Humanized anti-KC-4 Antibody

25 An anti-KC-4 fully humanized antibody was prepared by pairing fully humanized anti-KC-4 light and heavy chains by co-transfection as described in Example 69 above. The cell line, HuKC-4V2, was deposited with the ATCC on September 23, 1993 and awarded the accession number HB 11455.

Example 71:   Determination of Affinity Constants for Half
30   and Fully Humanized anti-KC-4 Antibodies

The secreted half humanized antibodies and the fully humanized antibody were purified from culture supernatants using a Sepharose 4B-protein A column (Bio-Rad, Richmond, CA.) as described by Ey et al. (Ey, P.L., et al. (1978), supra). Microtiter plates (Dynatech, Chantilly, VA) were prepared as described

35 by Ceriani et al. (Ceriani, R.L., et al. (1992), supra) using successive layers of

methylated BSA, glutaraldehyde, anti-β-galactosidase and the bacterial fusion protein 11-2 (a hybrid of β-galactosidase and human mammary mucin). Each well contained 388 ng of the 11-2 fusion protein. To each well were added 25 μl ^{125}I-KC-4 in RIA buffer (10 % bovine calf serum, 0.3% triton X-100, 0.05 %

5   sodium azide pH 7.4, in phosphate buffer saline) and compete with 25 μl of either unlabeled murine or chimeric antibody in RIA buffer at the final concentrations of 130 pM, 850 pM, 1.3 nM, 4 nM, and 13 nM). Iodinations were performed with ^{125}I (17 Ci/mg, Nordion International). 50 μg anti-KC-4 monoclonal antibody (Coulter, Hialeah, FL) were labeled at a specific activity of

10   9.56 mCi/mg using the chloramine T method as described previously by Ceriani et al. (Ceriani, R.L., et al. (1988), supra).

The antibody-antigen affinity constants were determined by taking the reciprocal of the concentration of competing unlabeled monoclonal antibody that produced 50 % binding as described by Sheldon et al. (Sheldon, K., et al.

15   (1987), supra). The protocol used to determine affinity constants was as described above except that in each case, an unlabeled antibody competed for binding to the antigen against the same radiolabeled antibody. The fully humanized antibody was shown to compete about as well or better with the anti-KC-4 murine antibody for the KC-4G3 antigen.

20   Polyacrylamide gel electrophoresis was performed to insure that the antibody chains migrated as expected. The affinity binding constants of the murine, chimeric, half humanized and humanized antibodies were determined in independent competition assays.

Example 72:   Histochemical Specificity of Half and Fully
25   Humanized anti-KC-4 Antibodies
Immunohistochemical staining using the immunoperoxidase technique of consecutive human breast carcinoma tissue sections was used as a test to verify that the analogue antibodies retain the affinity for the KC-4G3 carcinoma antigen of the murine antibody. Breast carcinoma tissue sections were stained with the

30   supernatant of the KC-4 murine and fully humanized transfected cells using the Vectastain ABC method (Vector Labs, Burlingame, CA). Both antibodies showed strong staining patterns.

The following Table 54 shows the results of the immunoperoxidase staining of five human breast carcinomas with either the standard anti-KC-4G3

35   murine or the fully humanized antibodies. Both stained the same tissues at a comparable level.

Table 54:   Immunoperoxidase Staining of Human Breast
Carcinoma Tissue Sections with Murine and
Fully Humanized anti-KC-4 Antibodies

Breast Tumor	Murine Antibody	Fully Humanized Antibody
1	+ +	+ +
2	+ + +	+ + +
3	-	-
4	+ +	+ +
5	+ + +	+ + +

Example 73:   Binding to HMFG of Half Humanized and
Fully Humanized anti-KC-4 Antibodies

Tissue culture supernatants from transfectants of all three anti-KC-4 variants of the humanized antibody were shown to bind the human milk fat globule (HMFG) as determined by radio-immunodetections.

Example 74:   Half Humanized and Fully Humanized
anti-KC-4 Antibodies Bind to Goat
anti-Human κ or γ Antibodies

Tissue culture supernatants from transfectants of all three variants of the anti-KC-4 humanized antibody were shown to bind in sandwich radioimmunodetections to both goat anti-human kappa chain antibody bound to microtiterplate wells (750ng/well), and to radio-iodinated ^{125}I-labeled goat anti-human gamma chain antibodies.

The results of these sandwich assays demonstrate that both chains of the humanized antibodies indeed possess human kappa and gamma constant regions.

Example 75:   Deduced Amino Acid Sequences of Humanized
anti-KC-4 Variable Light and Heavy Chains

The amino acid sequences of the light and heavy chains of the analogue humanized antibody are shown in Tables 55 and 56 below. The actual amino acid sequences may be varied either to increase affinity for the antigen or to decrease immunogenicity in humans. Numerous variants of this sequence may be engineered in accordance with the invention.

Table 55: Humanized anti-KC-4 Antibody V_L Analogue Sequence

---

anti-KC-4 V_L FR-HZ

MKLPVRLLVL MFWIPASSSD VLMTQTPLSL PVTPGEPASI SCRSSQSIVH

SNGNTYLEWY LQKPGQSPQL LIYKVSIRFS GVPDRFSGSG SGTDFTLKIS

5 RVEAEDVGIY YCFQGSHVPY TFGGGTKLEI K (Seq. ID No: 95)

Table 56: Humanized anti-KC-4 Antibody V_H Analogue Sequence

---

anti-KC-4 V_H FR-HZ

MDFGLSLVFL VLILKGVQCE VQMVESGGGL VQPGGSLRLS CAASGFAFSS

10 YAMSWVRQAP GKGLEWVAEI SSGGNYAYYQ DTVTGRFTIS RDNSKNTLYL

QMNSLRAEDT AVYYCAREDY GIPAWFAYWG QGTLVTVSS (Seq. ID No: 96)

Example 76: Half Humanized-Half Chimeric anti-BrE-3 Antibodies

A humanized BrE-3 light chain was paired with a non-humanized chimeric

15 BrE-3 heavy chain by co-transfection of SP2/0-Ag14 myeloma cells with hybrid

plasmids carrying the respective DNA sequences and those of a human F_c. The

cell line obtained, HuBrE3V1, was deposited with the ATCC on November 10,

1993 and awarded an accession number HB _____.

In addition, a humanized BrE-3 heavy chain was paired with a non-

20 humanized chimeric BrE-3 light chain as described in Example 69 above. The

thus obtained cell, HuBrE3V3 was deposited with the ATCC on November 10,

1993 and awarded an accession number HB _____.

Example 77: Fully Humanized BrE-3 Antibody

The fully humanized BrE-3 antibody was prepared by pairing fully humanized

25 BrE-3 light and heavy chains by co-transfection as described in Example 69

above. The thus obtained cell line, HuBrE3V2, was deposited with the ATCC on

November 13, 1992 and awarded the accession number HB 1111200.

Example 78: Half Humanized and Fully Humanized
           BrE-3 Antibodies Bind to Goat
30   anti-Human κ or γ Antibodies

Tissue culture supernatants from transfectants of all three variants of the

humanized BrE-3 antibody were shown to bind in sandwich

radioimmunodetections to both goat anti-human kappa chain antibody bound to

microtiterplate wells (750ng/well), and to radio-iodinated ^{125}I-labeled goat anti-human gamma chain antibodies.

The results of these sandwich assays demonstrate that both chains of the humanized antibodies indeed possess human kappa and gamma constant

5   regions.

Example 79: Binding to HMFG of Half Humanized and
             Fully Humanized BrE-3 Antibodies
Tissue culture supernatants from transfectants of all three BrE-3 variants of the humanized antibody were shown to bind the human milk fat globule (HMFG)

10   as determined by radio-immunodetections.

Example 80:   Competition Assays and Determination of Affinity
              Constants for Half Humanized BrE-3 Antibodies
The secreted half humanized antibodies were purified from culture supernatants using a Sepharose 4B-protein A column (Bio-Rad, Richmond, CA.)

15   as described by Ey et al. (Ey, P.L., et al. (1978), supra). The fully humanized antibody (HuBrE3 V2) was not purified.   Its concentration in the culture supernatant was determined by radioimmunodetection using a plate-bound goat anti-human kappa chain antibody as a capturing antibody and using radiolabled goat anti-human gamma chain antibody as a detecting antibody.   A parallel

20   standard curve of human $IgG_1\kappa$ was used to determine the unknown concentration of HuBrE3 V2. Microtiter plates (Dynatech, Chantilly, VA) were prepared as described by Ceriani et al. (Ceriani, R.L., et al. (1992), supra) using successive layers of methylated BSA, glutaraldehyde, anti-β-galactosidase and the bacterial fusion protein 11-2 (a hybrid of β-galactosidase and human

25   mammary mucin). Each well contained 388 nanograms of the 11-2 fusion protein. To each well were added 25 μl [125]I -BrE-3 in RIA buffer (10 % bovine calf serum, 0.3% triton X-100, 0.05 % sodium azide pH 7.4, in phosphate buffer saline) and competed with 25 μl of either unlabeled murine or humanized antibody in RIA buffer at the final concentrations of 130 pM, 850 pM, 1.3 nM,

30   4 nM, and 13 nM). Iodinations were performed with [125]I (17 Ci/mg, Nordion International). 50 μg BrE-3 monoclonal antibody (Coulter, Hialeah, FL) were labeled at a specific activity of 9.56 mCi/mg using the chloramine T method as described previously by Ceriani et al. (Ceriani, R.L., et al. (1988), supra).

All humanized versions of the BrE-3 antibody were shown to compete

35   about as well or better with the labeled murine BrE-3 antibody for the antigen as did the murine BrE3 antibody. The protocol used to determine affinity constants was as described above except that in each case, an unlabeled antibody competed for binding to the antigen against the same radiolabeled antibody. The antibody-antigen affinity constants were determined by taking the reciprocal of

the concentration of competing unlabeled monoclonal antibody that produced 50 % binding as described by Sheldon et al. (Sheldon, K., et al. (1987), supra).

Polyacrylamide gel electrophoresis was performed to insure that the antibody chains migrated as expected. The affinity binding constants of the
5   murine, chimeric, half humanized and humanized antibodies were determined in independent competition assays.

Example 81:   Histochemical Specificity of Half
              Humanized BrE-3 Antibodies
              Immunohistochemical staining using the immunoperoxidase technique of
10 consecutive human breast carcinoma tissue sections was used as a test to verify
that the analogue antibodies retain the affinity for the carcinoma antigen of the murine BrE-3 antibody. Breast carcinoma tissue sections were stained with the supernatant of cells transfected with the HuBrE3 V1 humanized antibody using the Vectastain ABC method (Vector Labs, Burlingame, CA). Both antibodies
15 showed strong staining patterns.

The following Table 57 shows the results of the immunoperoxidase staining of five human breast carcinomas with either the standard BrE-3 murine or the half humanized HuBrE3 V1 antibody. Both stained the same tissues at a comparable level.

Table 57 :   Immunoperoxidase Staining of Human Breast
                 Carcinoma Tissue Sections with Murine and
                 Half Humanized BrE-3 Antibodies

Breast Tumor	Murine Antibody	Half Humanized Antibodies
1	+ +	+ +
2	+ + +	+ + +
3	-	-
4	+ +	+ +
5	+ + +	+ + +

Example 82:   Hybridoma Cell Deposits
        The following cell lines were deposited as present examples of the best mode of the invention. The hybridoma cell lines expressing the murine-human fully chimeric BrE-3 and anti-KC4 antibodies and the fully humanized BrE-3 antibody were deposited with the ATCC on November 13,1992 under the Budapest Treaty, and have been assigned Accession Nos. HB 11199 (Chimeric BrE-3 A1C10), HB 11201 (Chimeric anti-KC-4 1E8) and HB 11200 (Humanized BrE-3 A1C10). The hybridoma cell line expressing the anti-KC-4 humanized

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antibody was deposited with the ATCC on September 23, 1993 and has been assigned Accession No. HB 11455 (Humanized HuKC-4 V2). The half chimeric/ half humanized antibody cell lines deposited with the ATCC are as follows: ATCC No. HB 11454 (light humanized/ heavy chimeric chains, HuKC-4V1) and ATCC No. HB 11456 (Heavy humanized/ light chimeric chains, HuKC-4V3) were deposited on September 23, 1993, and ATCC No. HB _____ (light humanized/ heavy chimeric chains, BrE3V1) and ATCC No. HB _____ (heavy humanized/ light chimeric chains, BrE3V3) were deposited on November 10, 1993 under the Budapest Treaty as examples of the best mode of the invention known to the inventors.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

WHAT IS CLAIMED AS NOVEL AND UNOBVIOUS AND
DESIRED TO BE PATENTED IN LETTERS PATENT IS:

1. A pure, isolated analogue peptide which selectively binds to an
antigen that is present on the surface or in the cytoplasm of a neoplastic
cell or that is released by the cell, the peptide being selected from the
group consisting essentially of at least one CDR of the light or heavy
chain of an antibody of a first species having affinity and specificity for
the human mammary fat globule (HMFG) antigen and an antigen found on
the surface or the cytoplasm of a neoplastic cell or that is released by the
cell; at least one variable region of the light or heavy chains of an
antibody of the first species having affinity and specificity for the HMFG
antigen and an antigen found on the surface or the cytoplasm of a
neoplastic cell or that is released by the cell, wherein about 1 to at least
46 amino acids in the FRs are substituted per chain with amino acids
selected from the group consisting of amino acids present in equivalent
positions in antibodies of a species other than the first species, or
fragments thereof comprising 1 to 3 CDRs per chain and flanking regions
thereof, each of about 1 to at least 10 amino acids, alone or with an N-
terminal fragment of about 1 to at least 10 amino acids; combinations
thereof, wherein each analogue peptide is operatively linked to at least
one other variable region peptide or analogue thereof; and mixtures
thereof.

2. The anti-neoplastic analogue peptide of claim 1, wherein the
antibody of the first species is selected from the group consisting of
murine, rat, goat, rabbit, canine, primate, guinea pig, bovine, equine,
feline and porcine antibodies.

3. The anti-neoplastic analogue peptide of claim 1, being able to
compete with the antibody secreted by the hybridoma cell ATCC No. HB
10028 for the human mammary fat globule antigen.

4. The antineoplastic analogue peptide of claim 1, having an
amino acid sequence selected from the group consisting of amino acid
Sequence ID Nos: 67 through 73; amino acid Sequence ID Nos: 75
through 81; fragments thereof comprising at least one CDR or one CDR
and two flanking regions thereof per chain; combinations thereof, wherein

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each analogue peptide is operatively linked to at least one other anal gue

peptide; and

mixtures thereof.

5. A glycosylated analogue peptide selectively binding to an antigen present on the surface or in the cytoplasm of a neoplastic cell or that is released by the cell, comprising the analogue peptide of claim 1; and at least one glycosyl residue operatively linked to the peptide.

6. A composition of matter, comprising the analogue peptide of claim 1; and a carrier.

7. A diagnostic kit for the in vivo therapy of neoplasms, comprising the composition of claim 6 in pharmaceutically-acceptable form; and instructions for its use.

8. The kit of claim 7, further comprising a molecule capable of selectively binding the analogue peptide and comprising an effector agent operatively linked thereto.

9. The kit of claim 7, wherein the effector agent comprises a molecule selected from the group consisting of therapeutic, immunogenic and diagnostic agents, radioisotopes, DNA monomers, RNA monomers, chemical linkers, transmitter molecules, combinations thereof, and combinations thereof with peptide and non-peptide polymers or copolymers.

10. An in vitro carcinoma diagnostic kit, comprising the composition of claim 6; a solid support having operatively linked thereto an antigen which selectively binds to the analogue peptide; and instructions for its use.

11. The kit of claim 10, further comprising a molecule capable of selectively binding the analogue peptide and comprising an effector agent operatively linked thereto.

12. The diagnostic kit of claim 10, further comprising anti-constant region immunoglobulins, protein G, protein A, other antibody binding molecules or binding fragments thereof.

13. A hybrid analogue polymer, comprising at least one analogue peptide of claim 1 and at least one effector agent operatively linked to the

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peptide; combinations thereof; and mixtures thereof.

14. The hybrid analogue polymer of claim 13, wherein the effector agent is selected from the group consisting of monomers, and non-peptide and peptide polymers other than the constant region of an antibody of the same species.

15. The hybrid analogue polymer of claim 13, wherein the effector agent comprises a radioisotope, an enzyme, or a phosphorescent or a fluorescent label.

16. The hybrid analogue polymer of claim 14, wherein the effector agent comprises a molecule selected from the group consisting of therapeutic, immunogenic and diagnostic agents, radioisotopes, phosphorescent and fluorescent agents, enzymes, DNA and RNA monomers, chemical linkers, transmitter molecules, combinations thereof, and combinations thereof with peptide and non-peptide polymers or copolymers.

17. The hybrid analogue polymer of claim 14, wherein the effector agent comprises a non-peptide polymer selected from the group consisting of ester, ether, vinyl, amido, imido, alkylene, arylalkylene, cyanate, urethane, and isoprene polymers, halogenated polymers, DNA and RNA polymers, copolymers thereof, and copolymers thereof with peptide polymers or monomers.

18. The hybrid analogue polymer of claim 14, wherein the effector agent comprises a peptide polymer selected from the group consisting of the constant region of antibodies or fragments thereof, the CDRs or variable regions of antibodies, whole antibodies, Fab and Fab', (Fab')$_2$, or antibody fragments of a species other than the first species, analogues thereof, hormones, enzymes, peptide transmitters, combinations thereof, and combination thereof with non-peptide polymers, copolymers or monomers.

19. The hybrid analogue polymer of claim 18, wherein the analogue peptide comprises amino acid sequences of non-human origin; and the peptide polymer comprises at least one constant region of the light or heavy chains of a human antibody or fragments thereof capable

of binding to anti-constant region immunoglobulins, protein G or protein A or ther antibody binding molecules.

20. The hybrid analogue polymer of claim 19, being selected from the group consisting of an analogue peptide comprising two heavy and two light chains, each light and heavy chain comprising at least one non-human CDR or analogue variable region peptide and at least one human constant region or fragment thereof; at least one human-non-human analogue Fab, Fab', or (Fab')$_2$ fragment thereof; fragments thereof;combinations thereof; and mixtures thereof.

21. The hybrid analogue polymer of claim 20, wherein each pair of the analogue heavy and light chains has a different predetermined specificity.

22. The hybrid analogue polymer of claim 13, wherein the analogue peptide and the effector agent are operatively linked by a polymer.

23. The hybrid analogue polymer of claim 13, being selected from the group consisting of at least one CDR or analogue variable region of the heavy chain of a first antibody of the first species or fragment thereof comprising at least one CDR and two flanking regions thereof and a first effector agent; at least one CDR or analogue variable region of the light chain of a second antibody of the first species or fragments thereof comprising at least one CDR and two flanking regions thereof, and a second effector agent, wherein each chain has a predetermined specificity; combinations thereof; and mixtures thereof.

24. The hybrid analogue polymer of claim 13, wherein at least one first pair of light and heavy chains comprising at least one CDR or analogue variable region or fragments thereof is linked to at least one second pair of the analogue light and heavy chains comprising at least one CDR or one analogue variable region or fragments thereof.

25.   An anti-carcinoma composition, comprising the hybrid analogue polymer of claim 13; and a carrier.

26. An in vivo carcinoma therapy and diagnosis kit, comprising the anti-carcinoma composition of claim 25 in pharmaceutically-

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acceptable form; and instructions for its use.

27. The kit of claim 26, further comprising a molecule capable of selectively binding the analogue peptide and comprising an effector agent operatively linked thereto.

28. The kit of claim 27, wherein the effector agent comprises a molecule selected from the group consisting of therapeutic, immunogenic and diagnostic agents, radioisotopes, DNA monomers, RNA monomers, chemical linkers, transmitter molecules, combinations thereof, and combinations thereof with peptide and non-peptide polymers and copolymers.

29. A diagnostic kit for carcinomas, comprising the anti-carcinoma composition of claim 25; a solid support having operatively linked thereto an antigen which specifically binds to the hybrid analogue peptide; and instructions for its use.

30. The kit of claim 29, further comprising a molecule capable of selectively binding to the hybrid analogue polymer, the molecule having an effector agent operatively linked thereto.

31. The diagnostic kit of claim 29, further comprising anti-constant region immunoglobulins, protein G, protein A, other antibody binding molecules or binding fragments thereof.

32. A method of determining the presence of neoplastic cells in a tissue, comprising contacting a tissue suspected of comprising neoplastic cells with the anti-neoplastic analogue peptide of claim 1 and allowing the analogue peptide to bind to antigen associated with any neoplastic cells present in the tissue to form an analogue peptide-cell antigen complex; and

detecting the presence of any complexes formed.

33. An in vitro method of determining the presence of neoplastic cells in a tissue comprising the method of claim 32, wherein the tissue is excised from a subject prior to being contacted with the analogue peptide in vitro.

34. An in vitro method of diagnosing a neoplasm comprising the method of claim 33, and comparing the results with a standard cut-off

value determined by c mparing results btained for normal and neoplastic samples, wherein a result above th cut- ff value is diagnostic of a neoplasm.

35. The in vitro method of diagnosing a neoplasm of claim 34, conducted by contacting a biological sample obtained from a subject suspected of having a neoplasm with a known amount of the anti-tumor analogue peptide of claim 1 in the presence of a solid supported antigen molecule that is selectively bound by the analogue peptide and allowing the formation of solid supported analogue peptide-antigen molecule complexes and analogue peptide-sample antigen complexes with any neoplastic cell antigen present in the sample; detecting any complex formed between the analogue peptide and the solid supported neoplastic antigen; and comparing the result obtained with the standard cut-off value.

36. An in vivo method of imaging a neoplasm present in a subject, comprising the method of claim 32, wherein a pharmaceutically-acceptable composition comprising an effective amount of the analogue peptide in radiolabeled form to reach the neoplasm and bind thereto is administered to a subject suspected of having a primary or metastasized neoplasm; and   the detection is of any binding of the labeled analogue peptide to the antigen on the surface or in the cytoplasm of the neoplastic cells.

37. An in vivo method of diagnosing the presence of a neoplasm in a subject comprising the method of claim 36, and comparing the results with a standard cut-off value determined by comparing results obtained for normal and neoplastic samples, wherein a result above the cut-off value is diagnostic of a neoplasm.

38. A method of inhibiting the growth or reducing the size of a primary or metastasized neoplasm in a subject comprising administering to a subject in need of the treatment an effective amount of the anti-tumor composition of claim 25 in pharmaceutically-acceptable form, wherein the effector agent comprises a therapeutic agent.

39. The method of claim 38, further comprising administering to

the subject a molecule capable of selectively binding to the analogue peptide and having an effector agent operatively linked thereto.

40. A method of purging neoplastic cells from a biological fluid, comprising the method of claim 32: and separating any analogue peptide-cell complexes present from the remainder of the fluid.

41. An ex vivo method of purging neoplastic cells from a biological sample obtained from a subject, comprising obtaining a biological sample from a subject suspected of having a neoplasm; conducting the method of claim 40 with the biological sample; and replenishing the purged biological sample to the subject.

42. An in vitro histochemical method of assessing the presence of neoplastic cells in a tissue, comprising obtaining from a patient suspected of having a neoplasm a tissue sample and preparing therefrom a tissue substrate; contacting the tissue substrate with the analogue peptide of claim 1 and allowing the analogue peptide to bind to any neoplastic cells present therein; and detecting the presence of any complexes formed.

43. A hybridoma cell expressing the analogue peptide of claim 1.

44. The hybridoma cell of claim 43, having the ATCC No. HB 11200.

45. The hybridoma cell of claim 43, having the ATCC No. HB 11455.

46. The hybridoma cell of claim 43, having the ATCC No. HB 11454.

47. The hybridoma cell of claim 43, having the ATCC No. HB 11456.

48. The hybridoma cell of claim 43, having the ATCC No. HB _____ (HuBrE3V1).

49. The hybridoma cell of claim 43, having the ATCC No. HB _____ (HuBrE3V3).

50. A composition, comprising the hybridoma cell of claim 43, and a diluent or carrier.

51. A pure, isolated analogue polydeoxyribonucleotide, comprising an analogue oligodeoxyribonucleotide encoding the analogue peptide of

claim 1.

52. The analogue polyde xyribonucle tide of claim 51, wherein the analogue oligodeoxyribonucleotide comprises a DNA sequence selected from the group consisting of DNA Sequence ID No: 64; DNA Sequence ID No: 65; fragments thereof encoding at least one CDR or one CDR and two flanking regions per chain; redundant DNA sequences thereof; combinations thereof; and mixtures thereof.

53. A hybrid vector, comprising a vector having the analogue polydeoxyribonucleotide of claim 51 operatively linked thereto.

54. The hybrid vector of claim 53, further comprising a polydeoxyribonucleotide comprising an oligodeoxyribonucleotide encoding an effector peptide, the effector peptide-encoding polydeoxyribonucleotide being operatively linked to the vector.

55. A transfected host cell, carrying the hybrid analogue vector of claim 53.

56. A pure, isolated analogue polyribonucleotide, comprising an analogue oligoribonucleotide encoding the analogue peptide of claim 1.

57. A method of producing an analogue peptide which specifically binds to an antigen on the surface or in the cytoplasm of neoplastic cell, or is released by the cell, comprising a) cloning the analogue polydeoxyribonucleotide of claim 40 into a vector to form a hybrid vector;

b) transfecting a host cell with the hybrid vector and allowing the expression of the analogue peptide; and c) isolating the analogue peptide or mixtures thereof.

58. The method of claim 57, further comprising site-specifically modifying the codon of at least one amino acid in the framework region of a non-human antibody prior to step a) to obtain the analogue polydeoxyribonucleotide.

59. The method of claim 57, wherein steps a) and b) are conducted by cloning analogue polydeoxyribonucleotides encoding analogue peptides selected from the group consisting of at least one CDR or analogue variable region of the first species or fragments thereof comprising at least one CDR and two flanking regions of the heavy or

light chains of the antibody of the first species; and the method further comprises d) allowing the expressed analogue peptides to interact with one another to form double chains.

60. A method of producing a hybrid analogue peptide comprising an analogue peptide which specifically binds to an antigen on the surface or in the cytoplasm of a carcinoma cell or to an antigen released by the cell and an effector peptide, the method comprising a) transfecting a host cell with the hybrid analogue vector of claim 54 and allowing the expression of the anti-carcinoma hybrid analogue peptide; and b) isolating the anti-carcinoma hybrid analogue peptide or mixtures thereof.

61. An anti-idiotype polymer, comprising polyclonal antibodies raised against anti-tumor antibodies or the analogue peptide of claim 1; monoclonal antibodies thereof capable of specifically binding the anti-neoplasm antibody; fragments thereof selected from the group consisting of CDRs, Fab, Fab', (Fab')$_2$, variable regions, and fragments thereof; analogues thereof selected from the group consisting of antibodies, Fab, Fab', (Fab')$_2$, and variable regions wherein about 1 to at least 46 amino acids in the FR are substituted per chain with amino acids selected from the group consisting of amino acids present in equivalent positions in antibodies of species other than the first species, or fragments thereof comprising 1 to 3 CDRs per chain and flanking regions thereof, each of about 1 to at least 10 amino acids, alone or with an N-terminal fragment of about 1 to at least 10 amino acids; combinations thereof, and combinations thereof with an oligopeptide comprising the amino acid sequence APDTRPAPG or fragments thereof comprising the TRP trimer hexamers thereof comprising the trimer, or tandem repeats thereof, wherein each analogue peptide is operatively linked to at least one other analogue peptide; and mixtures thereof.

62. A hybrid anti-idiotype polymer, comprising the anti-idiotype polypeptide of claim 61; and an effector agent operatively linked to the anti-analogue idiotype polypeptide.

63. An anti-carcinoma vaccine, comprising the anti-idiotype polypeptide of claim 61; and a pharmaceutically-acceptable carrier.

64. The anti-carcinoma vaccine of claim 63, in unit dose form.

65. A anti-tumor vaccination kit , comprising the vaccine of claim 63 and a diluent in separate sterile containers; and instructions for its use.

66. A method of vaccinating against neoplasms comprising administering to a subject an effective amount of the composition of claim 63.

67. The vaccination method of claim 66, wherein the anti-idiotype polypeptide is administered in an amount of about 0.1 to 5000.00 μg/kg body weight/dose.

68. A method of lowering the serum concentration of a circulating antibody or polypeptide that binds to an antigen on the surface or in the cytoplasm of a carcinoma cell or that is released by the cell, comprising administering to a subject in need of the treatment a pharmaceutically-acceptable composition comprising an amount of the anti-idiotype polypeptide of claim 61 effective to bind the circulating antibody or polypeptide and accelerate its clearance.

69. The method of claim 68, wherein the anti-ideotype peptide is administered in an amount of about 0.01 to 5.000.00 mg/kg body weight/dose.

70. A method of inhibiting the growth or reducing the size of a primary or metastasized carcinoma tumor in a subject, comprising administering to the subject an effective amount of a pharmaceutically-acceptable composition comprising an anti-carcinoma hybrid polymer comprising an effector agent selected from the group consist of radioisotopes and therapeutic drugs, and an anti-carcinoma polypeptide which specifically binds to an antigen on the surface or in the cytoplasm of a carcinoma cell or that is released by the cell; allowing the hybrid polymer to reach the tumor and the anti-carcinoma polypeptide to bind thereto; and administering to the subject a pharmaceutically-acceptable composition comprising an amount of the anti-idiotype polypeptide of claim 61 effective to bind any residual or unbound circulating anti-carcinoma hybrid polypeptide and accelerate the clearance of the hybrid polymer.

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(57) Abstract

An analogue peptide comprises the variable regions of the light or heavy chains of an antibody of a first species selectively binding to a tumor antigen, such as a carcinoma antigen, has 1 to 46 amino acids of the framework regions per chain substituted with amino acids such as those present in equivalent positions in antibodies of a species other than the first species, or fragments thereof comprising 1 to 3 variable region CDRs per chain and optionally flanking regions thereof of 1 to 10 or more amino acids, alone or with an N-terminal fragment of 1 to 10 or more amino acids, combinations or mixtures thereof. The polypeptide may also comprise an effector agent and/or be glycosylated, and is presented as a composition with a carrier. The analogue peptides are used in diagnostic kits for neoplasms such as carcinomas and methods for in vivo imaging and treating a primary or metastasized neoplasm such as a carcinoma, and in vitro diagnosing a neoplasm, ex vivo purging neoplastic cells from a biological fluid. RNAs and DNAs encode the analogue peptide, and a hybrid vector carrying the nucleotides and transfected cells express the peptides and a method produces the analogue peptide. An anti-idiotype polypeptide comprises polyclonal antibodies raised against an antineoplastic antibody or the analogue peptide of this invention, monoclonal antibodies thereof, Fab, Fab', (Fab')₂, CDR, variable region, or analogues or fragments thereof, combinations thereof with an oligopeptide comprising a TRP trimer, tandem repeats thereof, or combination or mixtures thereof. An anti-idiotype hybrid polypeptide with an effector agent and the anti-idiotype polypeptide, an anti-neoplastic vaccine, an anti-neoplastic vaccination kit, a method of vaccinating against neoplasms such as carcinomas and a method of lowering the serum concentration of a circulating antibody or polypeptide are provided.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5   C12N15/13   A61K47/48   C07K15/28   G01N33/574   C12N5/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5   C12N   A61K   C07K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 04380 (UNILEVER PLC) 19 March 1992 see the whole document ---	1-3,5,6, 50,51, 53,55-61
X	WO,A,90 12319 (JOHN MUIR CANCER & AGING INSTITUTE) 18 October 1990 see the whole document ---	4
X	WO,A,86 02945 (COULTER CORPORATION) 22 May 1986 see the whole document ---	4
P,X	EP,A,0 534 742 (CELLTECH LIMITED) 31 March 1993 see the whole document -----	1-6, 50-65

☐ Further documents are listed in the continuation of box C.   ☒ Patent family members are listed in annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet) (July 1992)

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark : Although claims 32, 36-39 and 66-70 are directed to a method of treatment of (diagnostic method practised on) the human body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)